EXPRESS MAIL NO. ***		
DECLARATION OF JAMES D. MURRAY UNDER 37 C.F.R. § 1.132	Attorney Docket Confirmation No.	UCDV-286 4027
	First Named Inventor	James D. Murray
	Application Number	10/663,454
	Filing Date	September 15, 2003
	Group Art Unit	1632
Address to: Commissioner for Patents Alexandria, VA 22313-1450	Examiner Name	J. Hama
	Title	*Stearoyl CoA desaturase transgenic non-human animals*

Dear Sir:

1. I, James D. Murray, declare and say I am a co-inventor of the claims of the above-identified patent application. I have over 20 years of experience in generating transgenic (non-human) animals. I have generated transgenic mice, goats, sheep, pigs, and cows.

2. I have read the Office Action dated April 6, 2005 in this application and understand that the Examiner has rejected pending claims 1, 3, 5-8, 13-21, and 31-43. In particular, the Examiner asserted that the specification does not reasonably provide enablement for a transgenic non-human mammal comprising a transgene encoding any fatty acid desaturase other than stearoyl CoA desaturase. The Examiner further asserted that the specification does not reasonably provide enablement for a method for producing a transgenic non-human mammal comprising introducing a desaturase transgene into a somatic cell, as recited in pending claim 18.

FATTY ACID DESATURASES

3. As of the September 17, 2002 priority date of the instant application, a large number of fatty acid desaturases were known and had been characterized enzymatically; and the nucleotide sequences encoding numerous such fatty acid desaturases were known. The instant specification provides the GenBank accession numbers of several nucleotide sequences encoding various fatty acid desaturases.

4. Stearoyl CoA desaturase (SCD) was chosen by as a model fatty acid desaturase, and SCD transgenic mice and goats were generated and characterized, as described in the instant patent application. However, one could readily generate a transgenic non-human mammal, as claimed, where the transgenic non-human mammal includes a transgene encoding any of a variety of fatty acid desaturases. For example, if a fatty acid desaturase-encoding nucleotide sequence were under transcriptional control of a mammary gland-specific promoter, one would reasonably expect that such a transgenic non-human mammal would produce milk having altered levels of the precursor fatty acids and downstream product fatty acids affected by the particular desaturase chosen. For example, one would reasonably expect that such a transgenic non-human mammal would exhibit higher levels of the product fatty acid in milk, compared levels of the fatty acid in milk of a non-transgenic mammal of the same species.

5. It is reasonable to expect that the results that we observed, using SCD as model fatty acid desaturase, would be observed using other fatty acid desaturases as transgenes. This is because the structure of a wide variety of fatty acid desaturases are known and the functional sites (e.g., catalytic domains) are conserved. See, e.g., the following reviews: Pereira et al., Prostaglandin, Leukotrienes and Essential Fatty Acids 68:97-106, 2003; Nakamura and Nara, Ann. Rev. Nutr. 24:345-376, 2004, copies of which are provided herewith as Exhibits 2 and 3. Function is conserved by maintaining the functional sites, even though the overall nucleotide and amino acid sequences are not necessarily highly conserved. For example, Table 1, below, shows that the level of nucleotide and amino acid sequence conservation of SCD of various species, compared to the human SCD, varies from about 47% to about 99% at the nucleotide (nt) sequence level and from about 53% to

about 99% at the amino acid (aa) sequence level, for SCD from eukaryotic species as divergent as the roundworm *Caenorhabditis elegans* to the chimpanzee. Nevertheless, in all cases the encoded protein is a functional stearoyl-CoA desaturase.

Table 1

Species	Gene	aa%ID	nt%ID
H.sapiens	**SCD**		
vs. P.troglodytes	LOC450676	99.1	99.5
vs. C.familiaris	LOC486839	90.5	89.4
vs. M.musculus	Scd1	85.6	83.1
vs. R.norvegicus	Scd1	85.4	83.6
vs. G.gallus	SCD	68.5	70.5
vs. A.gambiae	ENSANGP00000014991	58.7	56.9
vs. C.elegans	fat-5	47.0	53.5

6. Furthermore, recent work has demonstrated the ability of fatty acid desaturase genes from various eukaryotic species to function in transgenic plants or animals. The following examples illustrate this point. **Example 1**. The *Caenorhadbitis elegans* fat-1 (an omega 3 desaturase) and fat-2 (a delta-12 desaturase) coding regions produced functional desaturase protein when expressed in HC11 mouse mammary epithelial cells (Morimoto et al., *J. Dairy Sci.* 88:1142-1146, 2005; a copy of which is provided as Exhibit 4). **Example 2**. A *C. elegans* fat-1 transgene construct produced a functional omega-3 desaturase enzyme that resulted in altered tissue and milk fatty acid composition in transgenic mice constitutively expressing the transgene (Kang et al, *Nature* 427:504, 2004; a copy of which is provided as Exhibit 5). Thus, available data indicate that fatty acid desaturase coding regions from a variety of sources can be placed into transgene constructs and result in the production of functional desaturase proteins in a variety of eukaryotic species, including mammals.

METHODS FOR GENERATING TRANSGENIC NON-HUMAN MAMMALS

7. Claim 18 is directed to a method of generating a transgenic non-human

mammal, e.g., as recited in claim 1. The method recited in claim 18 is generally referred to as "somatic cell nuclear transfer." The method involves: a) introducing a fatty acid desaturase transgene into a mammalian somatic cell, forming a genetically modified somatic cell comprising a genetically modified nucleus; b) transferring the genetically modified nucleus from the genetically modified somatic cell into a single-celled embryo, generating a genetically modified single-celled embryo; and c) transferring the genetically modified single-celled embryo into a recipient female of the same species as the embryo, wherein the genetically modified embryo develops into a transgenic mammal in the female.

8. As of the September 17, 2002 priority date of the instant application, those skilled in the art knew how to generate a transgenic non-human mammal, using a somatic cell nuclear transfer method as recited in claim 18. There were various methods and gene constructions available to transform mammals, all of which have been extensively presented in publications and reviews. These include pronuclear microinjection, sperm-mediated gene transfer, retroviral or lentiviral vectors, and transformation of embryonic stem cells, primordial germ cells, or somatic cells with nuclei suitable for somatic cell nuclear transfer-based cloning. References are exhaustive, but a good recent technical publication is *Transgenic Animal Technology: A Laboratory Handbook 2nd Edition* edited by Carl Pinkert (Academic Press, ISBN 0-12-557166-6, 2002; "Pinkert"; Exhibit 6), which presents techniques that were widely available as of September 17, 2002. Pinkert summarizes and presents construct design, vector design as well as the methods for inserting a transgene construct into various mammalian species using somatic cell nuclear transfer-based methods. Practitioners skilled in the art would know Pinkert, and other texts, as well as the principle methodology of constructs design and transgene insertion pertinent to their species, including a wide range of mammals. A second book of a review nature that would cite many of the primary publications concerned with the art of making transgenic animals, including somatic cell nuclear transfer-based cloning approaches is *Transgenic Animals in Agriculture* (edited by JD Murray, GB Anderson, AM Oberbauer and MM McGloughlin, CABI Publishing, ISBN0-85199-293-5, 1999; "Murray"; Exhibit 7). Murray contains some of the original papers on somatic cell nuclear transfer-based cloning.

ACTIVITY OF STEAROYL COA DESATURASE

9. In the April 6, 2005 Office Action, the Examiner stated that SCD cannot catalyze the formation of fatty acids with more than one double bond, to generate linoleic acid. Office Action, page 7. However, SCD can in fact catalyze the formation of fatty acids with more than one double bond, to linoleic acid. An example of a CLA that can be generated by the action of SCD is C18:2 *cis-9 trans-11* fatty acid, whereby rumenic acid (18:1 trans-11) produced by rumen bacteria is acted upon by SCD in the tissues of ruminant animals, such as dairy cattle, to produce the CLA 18:2 *cis-9 trans-11* (Griinari et al., *J. Nutr.* 130:2285-2291, 2000; Corl et al., *J. Nutr. Biochem.* 12:622-630; copies of which are provided as Exhibits 8 and 9, respectively). The data presented in the instant application demonstrated that the level of CLA is increased in SCD transgenic goats. See, e.g., Figure 1C of the instant application.

10. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title XVIII of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

29 Nov 2005
Date

James D. Murray

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U.S. Patent Application No. 10/663,454
Exhibit 2

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Recent advances in the study of fatty acid desaturases from animals and lower eukaryotes

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Abstract

The biosynthesis of polyunsaturated fatty acids (PUFAs) in different organisms can involve a variety of pathways, catalyzed by a complex series of desaturation and elongation steps. A range of different desaturases have been identified to date, capable of introducing double bonds at various locations on the fatty acyl chain. Some recently identified novel desaturases include a Δ4 desaturase from marine fungi, and a bi-functional Δ5/Δ6 desaturase from zebrafish. Using molecular genetics approaches, these desaturase genes have been isolated, identified, and expressed in variety of heterologous hosts. Results from these studies will help increase our understanding of the biochemistry of desaturases and the regulation of PUFA biosynthesis. This is of significance because PUFAs play critical roles in multiple aspects of membrane physiology and signaling mechanisms which impact human health and development.

1. General introduction

Enzymes that catalyze the addition of a double bond (unsaturation) in a fatty acyl chain are categorized as fatty acid desaturases. This family of enzymes is found almost universally in most living cells, where they help regulate the fluidity of membrane lipids, and also play a critical role in the biosynthesis of PUFAs. These PUFAs serve as precursors for a number of biologically active molecules like eicosanoids, pheromones, growth regulators, and hormones. In mammals, eicosanoids like prostaglandins, leukotrienes, and thromboxanes act locally through autocrine or paracrine processes on G-protein linked cell surface receptors. This leads to the activation of various signaling mechanisms that have effects on numerous cellular functions including chemotaxis, vascular permeability, inflammation, vasoconstriction, etc. [1]. Thus, modulation of PUFA biosynthesis is a major target in the treatment of certain chronic diseases like arthritis, diabetes, inflammation, cancer, cardiovascular disease, etc.

The desaturation reaction catalyzed by the fatty acid desaturases is an aerobic process utilizing molecular oxygen and reducing equivalents (electrons) obtained from an electron transport chain. There are three types of fatty acid desaturases: acyl-CoA, acyl-lipid, and acyl-ACP desaturase. The acyl-CoA desaturases are membrane-bound enzymes that desaturate fatty acids esterified to Coenzyme A (CoA). These are present in animal, yeast and fungal cells. The acyl-ACP desaturases are found in plant plastids in a soluble form, and desaturate fatty acids linked to an acyl carrier protein (ACP). The acyl-lipid desaturases introduce unsaturated bonds in lipid-bound fatty acids. These are membrane-bound and found in plants, fungi, and cyanobacteria. The desaturase enzymes can be further categorized based on the electron donor used (cytochrome *b*5 versus ferredoxin) with their respective reductases, and either NADH or NADPH. The acyl-CoA desaturases of animals, and the acyl-lipid desaturases of plants and fungi generally use cytochrome *b*5 as the electron donor.

Desaturase enzymes are specific to the location, number and stereochemistry of double bonds already present in fatty acids [2]. Thus for example, a Δ9 desaturase can introduce a double bond only between

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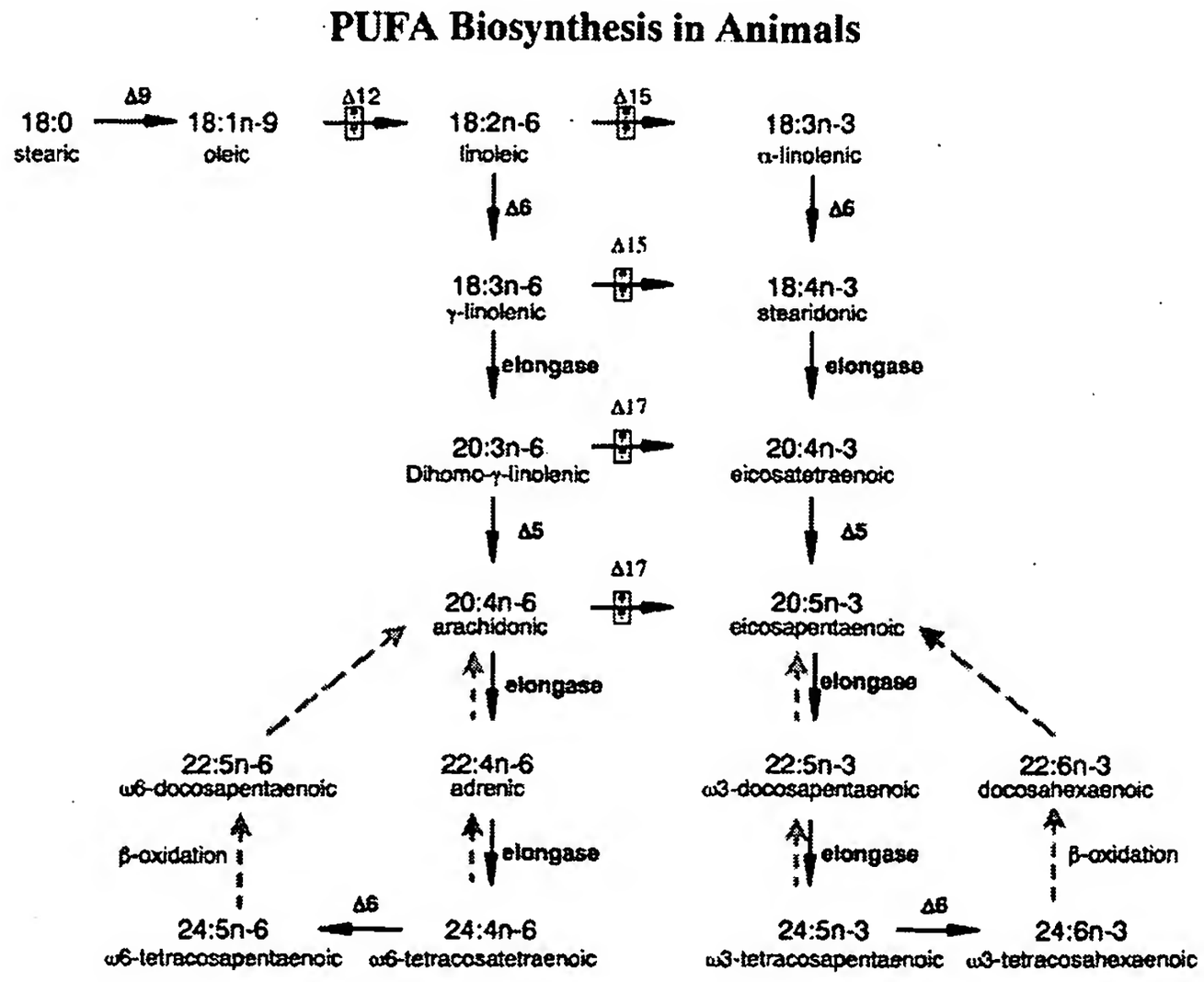

Fig. 1. Biosynthesis pathway of long-chain PUFAs in animals. The common pathway for synthesis of n-6 and n-3 long-chain fatty acids is shown in bold arrows and retroconversion is shown in dashed, gray arrows. Cross marks indicate the absence of those desaturates in animals.

carbon #9 and #10 of the fatty acid, wherein the carbon atoms are numbered from the carboxyl carbon (carbon #1). In the process of biosynthesis of long-chain polyunsaturated fatty acids (PUFAs), these different desaturases, along with a family of enzymes called elongases, introduce a consecutive series of desaturations and elongations of the fatty acyl chain to generate PUFAs like arachidonic acid (AA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) (Fig. 1).

This review focuses on current information available on membrane-bound desaturases from animals and lower eukaryotes, together with their expression studies in heterologous hosts. For discussions on plant and cyanobacterial desaturases, readers are referred to other excellent published reviews [3–5].

2. PUFA biosynthesis in animals

2.1. Introduction

Animals are capable of desaturating stearic acid (18:0) to oleic acid (18:1n-9) by means of a Δ9 desaturase enzyme. However, with certain exceptions, animals in general lack the Δ12 and Δ15 desaturases, which are required for the production of linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3:n-3). Thus, these fatty acids are considered to be essential and must be derived from the diet. The essential fatty acids can be further desaturated and elongated to varying degrees depending on the animal species, Δ6 and Δ5 desaturase enzyme activity and tissue location (Fig. 1). For example, cats appear to lack or express relatively low Δ6 (and possibly Δ5) desaturase activity [6]; marine fish such as turbot, gilthead, sea bream, and golden gray mullet have functional Δ6 desaturases, but display limited Δ5 desaturase activity [7,8]; whereas, freshwater fish like trout, carp and tilapia have functional Δ5 and Δ6 desaturases and can convert essential fatty acids to AA, EPA, and DHA [9]. Certain tissues in mammals tend to have higher concentrations of DHA than others (e.g. brain and retina) possibly due to higher expression of desaturase genes involved in DHA production [10].

The pathway leading to the biosynthesis of DHA from docosapentaenoic acid (DPA, 22:5n-3) has only recently been deciphered in mammals. Until 1991, it was postulated that a Δ4 desaturase catalyzed the conversion of DPA to DHA in the microsome. However, radiolabeling studies in mammalian cells have revealed a modified pathway for DHA synthesis [11,12]. Here, DPA is elongated to C24:5n-3, which is then desaturated by a Δ6 desaturase to generate C24:6n-3 in the microsome. C24:6n-3 is then thought to be transported to the peroxisomes, wherein it undergoes β-oxidation (retroconversion) to generate DHA (Fig. 1). The exact enzymes or specific mechanism involved in retroconversion are still not known, and there are speculations that retroconversion can also occur in the mitochondria [13–15].

2.2. Desaturases in animals

2.2.1. Stearoyl CoA (Δ9) desaturase (SCD)

The stearoyl CoA (Δ9) desaturase is one of the best-studied desaturases to date. This enzyme catalyzes the first step in the PUFA biosynthetic pathway, namely the incorporation of a double bond at carbon #9 of stearic acid to generate oleic acid. This enzyme is a microsomal membrane-bound protein and functions in conjunction with cytochrome *b*5 and NADH-dependent cytochrome *b*5 reductase. Both the SCD gene and the protein have been isolated from rat, which has facilitated biochemical characterization of the enzyme [16]. The SCD gene has also been isolated from several different species including mouse, human, fish and insects [5]. All these desaturases reveal the presence of two long hydrophobic domains capable of spanning the lipid bilayer twice, and three conserved histidine-box motifs containing eight histidine residues with the general structure $HX_{(3-4)}H$, $HX_{(2-3)}HH$, $HX_{(2-3)}HH$. Each of these histidine residues is essential for the catalytic activity of this enzyme as demonstrated by site-directed mutagenesis studies [17].

Since the SCD is the rate-limiting enzyme in cellular synthesis of monounsaturated fatty acids, it plays an important role in maintaining membrane fluidity. Alterations in the ratio of saturated to unsaturated fatty acids have been implicated in various disease states. The activity of SCD is sensitive to dietary changes, hormonal imbalance, peroxisome proliferators, etc. [18]. Elevated SCD activity is shown to be associated with obesity, and a positive correlation between SCD activity in skeletal muscle and percentage of body weight has been reported in humans [19,20]. Higher oleic acid levels, and hence high SCD activity, is reported to be associated with colorectal and mammary tumors, as well as colonic and esophageal carcinomas [21,22]. In addition, increase in SCD activity is correlated with cardiovascular diseases, because an increase in oleic acid in the liver causes a subsequent increase in exported cholesterol in form of VLDL, thus increasing plasma lipids and lipoproteins.

2.2.2. Δ12 desaturase and ω-3 desaturase

Unlike plants, most animals are incapable of desaturating C18 acyl chains at the Δ12 and Δ15(ω-3) position. However, some invertebrates have demonstrated the ability to introduce a double bond at the Δ12 and/or ω-3 (the third carbon atom from the ω carbon/methyl end of the fatty acyl chain) position on the fatty acid. Δ12 desaturase activity has been demonstrated in axenic tissues from the American cockroach (*Periplaneta americana*) and the house cricket (*Acheta domesticus*) [23,24], although the genes have not been characterized. This activity was localized in the endoplasmic reticulum, and utilized NADPH and CoA-linked fatty acids as substrate, in contrast to the acyl-lipid Δ12 desaturases in plants.

The nematode, *Caenorhabditis elegans*, can synthesize C20 PUFAs from diets containing only saturated fatty acids indicating the presence of Δ12 desaturase and/or Δ15 (ω-3) desaturase. A subsequent search of the *C. elegans* genome database reveled the presence of two desaturase-like genes, *fat1*, and *fat2* [25,26]. The *fat2* gene encodes a functional Δ12 desaturase that recognizes C18 as well as C16 substrates in a yeast expression system [26]. This predicted 376 residue protein contains the three conserved histidine-box motifs ('HXXXH', 'HXXHH', and 'HXXHH'), and two long hydrophobic domains, typical of all membrane-bound desaturases. Like all the plant Δ12 desaturases, this protein does not contain a fused cytochrome *b*5 domain. Thus, it is assumed that it interacts with a separate cytochrome *b*5 for its activity. It is not known if this enzyme is an acyl-CoA desaturase as seen in insects. In addition, this protein contains the 'KAKKAQ' sequence at its carboxyl terminus, thought to represent an endoplasmic reticulum retention signal present in many transmembrane proteins.

The *C. elegans fat1* encodes a novel ω-3 desaturase capable of introducing a double bond at the ω-3 position of both C18 as well as C20 fatty acids [25]. This is the first known ω-3 desaturase that can act on C20 PUFAs. This desaturase shows 32–35% identity with the Δ12 desaturase (FAD2), and the Δ15(ω-3) desaturase (FAD3) from *Arabidopsis*. The predicted 402 residue protein demonstrates all the characteristic features of a membrane-bound desaturase. These include the three histidine-rich motifs, and the two long stretches of hydrophobic residues that serve as the transmembrane domains. This protein is thought to be an acyl-lipid desaturase like known plant ω-3 desaturases.

fat1 has been expressed in mammalian cells [27,28]. Adenovirus mediated transfer of *fat1* into rat cardiac myocytes resulted in a change in n-6 to n-3 PUFA ratio from 15:1 to 1:1. In addition, the level of prostaglandin E2, an eicosanoid derived from arachidonic acid, was significantly reduced in the transgenic cell. Since deficiency of n-3 PUFAs is thought to be associated with an increase in the incidence of chronic disease conditions like cardiovascular disease, diabetes, obesity, etc., these findings are significant in terms of potential applications in human gene therapy. *fat1* has also been expressed in human breast cancer cells, which resulted in a dramatic decrease in the n-6:n-3 PUFA ratio [28]. The gene transfer also induced mass cell death and inhibited cell proliferation. This data is valuable in understanding the effects of n-3 PUFAs on cancer prevention and treatment.

2.2.3. Δ6 desaturase

Δ6 desaturase is a membrane-bound, acyl-CoA desaturase found in the endoplasmic reticulum of animals. It catalyzes the rate-limiting conversion of essential fatty acids to long-chain PUFAs. This enzyme desaturates LA to γ-linolenic acid (GLA, 18:3n-6), and ALA to stearidonic acid (STA, 18:4n-3). The Δ6 (and Δ5) desaturases are classified as 'front-end' desaturases because they are capable of introducing a double bond between a pre-existing double bond and the 'front'/carboxyl end of the fatty acid. These enzymes differ from the other desaturases in that they contain a fused cytochrome *b*5 domain at the N-terminus, which plays a role as an electron donor during desaturation. The fused N-terminal cytochrome *b*5 domain is essential for activity as demonstrated by site-directed mutagenesis studies. Loss of the histidine residue of the 'HPGG' conserved heme-binding domain of the cytochrome *b*5 region results in complete loss of enzyme activity [29].

As with other membrane-bound desaturases, the Δ6 desaturase has a tripartite motif comprised of a group of eight conserved histidines: $HX_{(3-4)}HX_{(7-41)}HX_{(2-3)}HHX_{(61-189)}HX_{(2-3)}HH$ [30]. However, in front-end desaturases, the first histidine residue of the third histidine-box motif is replaced by a glutamine (i.e. $QX_{(2-3)}HH$ instead of $HX_{(2-3)}HH$). This variant glutamine in the third histidine box is essential for catalytic activity, and cannot be replaced by histidine [31].

Δ6 desaturase genes have been identified from various mammals including human, rat, mouse [32,33], and also from the nematode, *C. elegans* [34]. All three mammalian Δ6 desaturases are predicted to have 444 amino acids, with the mouse and human sequences having 87% sequence homology. In addition, the Δ6 desaturase protein has been partially purified from rat liver microsome [35]. However, not much progress has been made since in terms of biochemical characterization of this enzyme. The human and rat Δ6 desaturases have recently been shown to have dual function when expressed in yeast [36,37]. Co-expression of the human C20/C22 elongase and human Δ6 desaturase demonstrated that a single D6 desaturase is capable of functioning on both C18 as well as C24 fatty acids. Thus, this Δ6 desaturase can convert: LA to GLA; ALA to STA; C24:4n-6 to 24:5n-6; and C24:5n-3 to 24:6n-3. The C24 fatty acids are intermediates involved in the synthesis of DHA (Fig. 1).

The mouse and human Δ6 desaturase genes have been functionally expressed in rat hepatocytes and Chinese hamster ovary cells (CHO), and could convert LA to GLA and ALA to STA in vivo. Northern analysis has revealed that expression of the Δ6 desaturase is the greatest in the human liver, followed by brain, heart, and lung. Differences in transcript levels are consistent with differences in Δ6 desaturase enzymatic activity that reportedly exists among various tissues, especially the liver and the brain [38]. The activity of the D6 desaturase in vivo is regulated by certain dietary components, age, and hormones [39]. In addition, in chronic diseases like cancer and diabetes, altered expression levels of this enzyme in different tissues have been observed [40–46].

2.2.4. Δ5 desaturase

The Δ5 desaturase catalyzes the final step in the production of the C20 PUFAs AA and EPA. This desaturase is also considered a front-end desaturase and shares all the conserved structural characteristics displayed by other front-end desaturases like the Δ6 desaturase. Δ5 desaturase genes have been identified from several animals including human, rat, and *C. elegans* [47–49]. The rat Δ5 desaturase appears to recognize both CoA-linked and glycerolipid-linked acyl substrates, in contrast to most animal desaturases that recognize only CoA-linked substrates [50,51]. The *C. elegans* Δ5 desaturase is different in that it is capable of inserting double bonds in non-methylene interrupted fatty acids such as 20:2Δ11, 14,20:3Δ11,14,17, and yeast endogenous 18:1, in addition to desaturating its regular C20 PUFA substrates [49].

The human Δ5 desaturase is predicted to have 444 amino acids, and shares 62% identity with the human Δ6 desaturase. Expression analysis of human Δ5 desaturase in CHO cells and mouse L-cells has confirmed that this enzyme can convert dihomo-γ-linolenic acid (DGLA, 20:3n-6) to AA, and eicosatetraenoic acid (ETA, 20:4n-3) to EPA [47]. In fact, the expression of this desaturase in mammalian cells results in an increase in longer chain fatty acids downstream of the PUFA synthesis pathway, including adrenic acid (ADA, 22:4n-6), EPA and DHA. Northern analysis has revealed that expression of Δ5 desaturase is greatest in the adrenal gland, followed by liver, and brain. This is consistent with the highest level of ADA being found in the adrenal gland. The activity of the Δ5 desaturase is regulated by diet [52], and altered expression levels of this enzyme have been associated with various disease conditions including eye disorders, Alzheimer's disease, and diabetes [53–55].

2.2.5. Bi-functional Δ5/Δ6 desaturase

Several freshwater fish species like trout, carp, and zebrafish can convert dietary C18 fatty acids to their corresponding C20 and C22 PUFAs [9], unlike some of the marine fish like turbot and sea bream [56,57]. Thus, these freshwater fish are predicted to contain both Δ5 and Δ6 desaturases. Recently, a novel desaturase gene has been characterized from zebrafish (*Danio rerio*) which exhibits both Δ6 and Δ5 desaturase activity [58]. The predicted protein of this bi-functional Δ5/Δ6 desaturase contains 444 residues, and displays 64%

amino acid identity with human Δ6 desaturase, and 58% identity with the human Δ5 desaturase. This protein displays all the characteristics of microsomal membrane-bound fatty acid desaturases, including the presence of three conserved histidine-box motifs, two long transmembrane hydrophobic domains, and a fused N-terminal cytochrome *b*5 region. This protein is more active on Δ6 desaturase substrates (29.4% conversion of ALA to STA) than with Δ5 desaturase substrates (20.4% conversion of ETA to EPA). In addition, it showed a preference for the conversion of n-3 fatty acids over the n-6 fatty acids.

From a phylogenetic standpoint, it is thought that this bi-functional desaturase is a component of the prototypic vertebrate PUFA biosynthetic pathway that persisted in the freshwater fish species. The evolution of two distinct enzymes for Δ5 and Δ6 desaturation could be an adaptive response to terrestrial diets that provided relatively lower amounts of preformed C20 and C22 PUFAs, as compared to the diets obtained by freshwater fish. In line with this, desaturase sequences related to this bi-functional Δ5/Δ6 desaturase have been identified in other freshwater fish like trout and carp, although the functionality of these still needs to be determined.

3. PUFA biosynthesis in lower eukaryotes

3.1. Introduction

A number of lower eukaryotes such as fungi, algae, and protozoa are known to produce large amounts of PUFAs with chain lengths of C20 or greater [59]. Thus, these organisms are predicted to contain the complete array of enzymes necessary for the biosynthesis of EPA and DHA from stearic acid (Fig. 2). In addition, some lower eukaryotes display an alternate pathway for C20 PUFA production involving a D8 desaturase, as well as the conventional PUFA pathway [60–62].

Most of these high EPA- and DHA-producing organisms tend to be microalgae or fungi that inhabit colder marine environments. These organisms can produce DHA at levels ranging from 2% to 30% of their total fatty acids [59]. Some of the high DHA producers include the dinoflagellate, *Crypthecodinium cohnii*, and the heterokont algae, *Thraustochytrid* [59,63]. These microalgae serve as a primary food source and major source of PUFAs for fish and other sea life. Some of these microalgae are currently being used to generate 'designer oils' that contain either AA, EPA or DHA as the sole bioactive fatty acid [64,65], and optimization of PUFA production by fermentation manipulations are currently underway [66]. For example, the fungus *Mortierella alpina* can produce ~38% oil containing solely n-6 PUFAs, and is being used to generate AA-rich oils [67]. In addition, some microalgae like *Isochrysis* and *Nannochloropsis* are used to enrich rotifers with EPA and DHA, which are then used in aquaculture to enrich the diets of fish and shrimp [68].

In these DHA-rich organisms, the final step leading to the production of DHA appears to be distinct from that in animals, and has not been fully resolved. In *Thraustochytrids*, a Δ4 desaturase was characterized, which was capable of converting DPA to DHA [69]. However, radiolabeling studies in these organisms have indicated that DHA biosynthesis may occur via the prokaryotic polyketide synthase (PKS) pathway [70] similar to that seen in EPA-/DHA-producing bacteria like *Shewanella* sp. and *Vibrio* sp. [71,72]. Whether the desaturase pathway, or the PKS pathway, or both, functions in DHA production in these lower eukaryotes remains to be resolved.

3.2. Desaturases from lower eukaryotes

3.2.1. Stearoyl CoA (Δ9) desaturase

The stearoyl CoA (Δ9) desaturase from fungi and animals are similar in that they are microsomal membrane-bound proteins, and require oxygen, cytochrome *b*5, as well as NADH-dependent cytochrome *b*5 reductase for activity. Δ9 desaturase genes have been isolated from several species including *Saccharomyces cervisiase*, *M. alpina*, *Histoplasma capsulatum*, and *Tetrahymena thermophila* [5,73,74]. Although these desaturases do not share high amino acid identity with the animal counterpart, they share the same structural motifs including the three histidine-box motifs, and the two hydrophobic domains that can traverse the phospholipid bilayer.

The yeast Δ9 desaturase is unique in that it contains a cytochrome *b*5 domain at its C-terminus, and this domain is required for the functional activity of this protein [75]. This has also been observed in an algal Δ9 desaturase gene isolated from the red alga *Cyanidioschyzon merolae* [76].

3.2.2. Δ12 desaturase and ω-3 desaturase

Δ12 desaturase genes have been characterized from the oleaginous fungus *M. alpina* [77,78], *Mucor rouxii* [79], and the green alga, *Chalamydomonas reinhardtii* [80]. These Δ12 desaturases are highly similar, in terms of sequence identity, to the animal Δ12 desaturases. The *M. alpina* Δ12 is highly active in yeast, resulting in 71.4% conversion of the exogenously added oleic acid substrate to linoleic acid. Δ12 desaturase activity has been characterized in the non-photosynthetic protist, *Acanthamoeba castellanii* [81], and the yeast, *Lipomyces starkeyi* [82].

The *M. alpina* Δ12 desaturase has been expressed in mouse L-cells [83]. Mammalian cells typically do not possess a Δ12 desaturase, and hence have a requirement

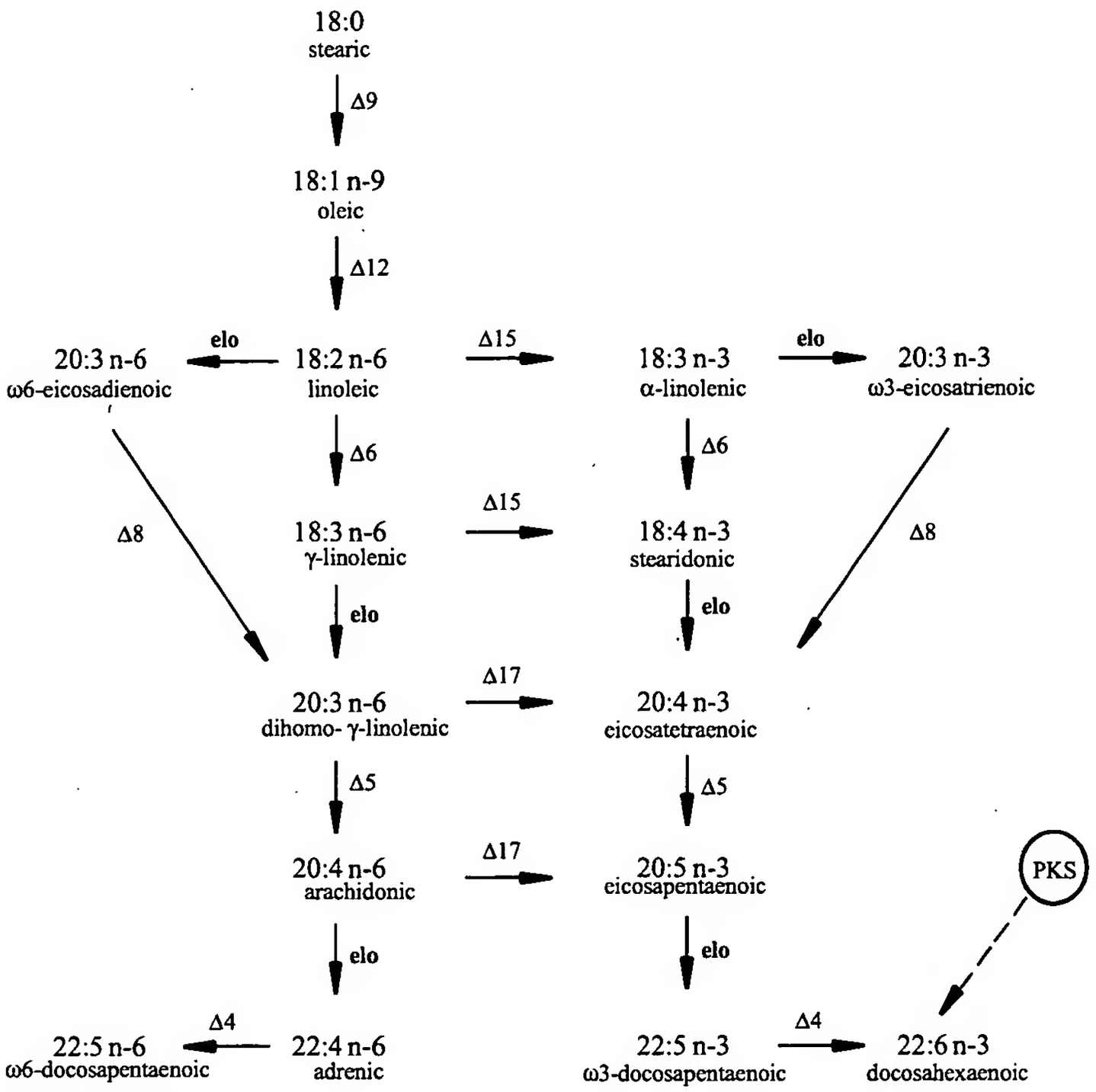

Fig. 2. Biosynthesis pathway of long-chain PUFAs in lower eukaryotes. The common pathway for synthesis of n-6 and n-3 long-chain PUFAs are shown in bold arrows, and the alternate PKS pathway that exist in some organisms is indicated in dashed arrow.

for essential fatty acids. However, expression of the Δ12 desaturase gene in L-cells resulted in a significant increase in the amount of LA, most of which was incorporated into cellular phosphatidylcholine. In addition, there was a corresponding increase in endogenous PUFAs like DGLA and AA. An increase in eicosadienoic acid (20:2n-6) was also observed suggesting the presence of an elongase that acts directly on 18:2n-6. These recombinant L-cells could survive for a limited period of time on serum-free media. However, prolonged incubation of these transgenic L-cells resulted in a dramatic decrease in the production of DGLA and AA, which might be indicative of the regulatory effect of high LA levels on the activity of endogenous Δ6 desaturase. These experiments have thus demonstrated the possibility of generating mammalian cell lines with no requirements for exogenous essential fatty acids, which can be used for further studies on the PUFA pathway regulation and metabolism in vivo.

Although no fungal or algal $\omega-3$ desaturase has been published so far, this enzyme is predicted to exist in some species of filamentous fungi that exhibit high AA and EPA levels [59,84].

3.2.3. Δ6 desaturase

Fungal Δ6 desaturases has been identified from *M. alpina* [77] and *Mucor rouxii* [85]. The predicted proteins display all the characteristics of microsomal membrane-bound, front-end fatty acid desaturases. The *M. alpina* Δ6 desaturase has been functionally expressed in yeast, as well as in *Aspergillus oryzae* [77,78]. The *Mucor rouxii* Δ6 desaturase differs from that of *M. alpina* in that it is larger (523 amino acids), and is more similar in sequence to plant Δ6 desaturase than to fungal or animal Δ6 desaturases. This desaturase contains an unusual histidine-rich motif 'HKHHSH' downstream of the cytochrome *b*5 domain, lying within the region of the amino acid sequence that is absent in other Δ6

desaturases. This region is thought to be essential for enzyme activity [85]. Δ6 desaturase activity has also been demonstrated in microsomal extracts from yeast *Torulopsis* and *Candida lipolytica* [51], and was capable of acting on both CoA-linked and glycerolipid-linked fatty acid substrates. This is in contrast to the Δ6 desaturase from *Mucor circinelloides* that acts exclusively on glycerolipid-linked LA [86].

The *M. alpina* Δ6 desaturase gene has been functionally expressed in mammalian cells [83]. In this study, the insertion of the gene in mouse L-cells resulted in an increase in the endogenous levels of DGLA and AA. Therefore, through genetic modifications it is possible to manipulate the fatty acid biosynthesis pathway in vivo, to enhance the production of PUFAs and their derivatives.

Functional expression of the *M. alpina* desaturase genes in plants has demonstrated their viability in generating PUFA-rich oilseeds. Here, the *M. alpina* Δ6 and Δ12 desaturase genes were co-expressed in a low linolenic variety of *Brassica napus*. These gene were shown to be simultaneously expressed, and resulted in the accumulation of >40% GLA in the transgenic canola oil [87].

3.2.4. Δ5 desaturase

Δ5 desaturase genes have been characterized from *M. alpina* [88,89], as well as the heterokont algae *Thraustochytrium* [69], and functionally expressed in yeast. The predicted proteins display all the characteristic motifs seen in the animal Δ5 desaturases. The *M. alpina* Δ5 desaturase is also capable of producing taxoleic acid (Δ5,9-18:2) and pinolenic acid (Δ5,9,12-18:3) when introduced to low linolenic variety of *B. napus*, demonstrating its ability to function as a Δ5 desaturase in higher plants.

In the slime mold *Dictyostelium discoideum*, two Δ5 desaturase genes have been identified [90,91]. These two genes are 66% identical to each other, and their predicted proteins display 38.6–42% sequence identity with the *M. alpina* Δ5 desaturase. Although they contain all the characteristic features of a membrane-bound, front-end desaturase, functional analysis in yeast revealed that they can add a double bond at the Δ5 position of C16 and C18 monoenoic acids only, thus indicating that they are not involved in long-chain PUFA metabolism.

3.2.5. Δ8 desaturase

In some eukaryotes like *Euglena gracilis*, an alternate pathway for the synthesis of C20 PUFAs exists. Here, the conventional Δ6 desaturation/elongation step in the conversion of LA to DGLA is bypassed, and replaced by an alternate pathway involving a novel Δ8 desaturase [60]. In this alternate pathway, LA is elongated to 20:2n-6, which is acted upon by the Δ8 desaturase to generate DGLA (Fig. 2). This pathway also functions on n-3 PUFAs. Δ8 desaturase activity has also been demonstrated in the ciliated protozoan, *Tetrahymena* [92], and the soil amoeba, *Acanthamoeba* [93]. In the alga *Isochrysis*, although a D8 desaturase gene has not been identified, there exist a C18-elongase that specifically converts LA to 20:2n-6, and ALA to 20:3 n-3 [62], indicative of a functional Δ8 desaturase pathway.

The Δ8 desaturase shares similar features with the front-end desaturases; it contains a cytochrome *b*5 domain at its N-terminus, as well as the three conserved histidine-box motifs of which the third histidine-box has the 'QXXHH' motif. The predicted protein shares only 33% identify with the *C. elegans* front-end desaturases, and 28% identity with the borage Δ6 desaturase.

3.2.6. Δ4 desaturase

A Δ4 desaturase was recently identified from *Thraustochytrium*, a heterokont alga that produces a large amount of DHA(~30% of total fatty acid) [69]. Prior to this discovery, the existence of this enzyme was questionable, especially since DHA production in mammals was found to involve D4 desaturase-independent pathway (refer Section 2.1). The Δ4 desaturase is a front-end desaturating enzyme capable of introducing a double bond at carbon #4 of $\omega-3$ docosapentaenoic acid (DPA, 22:5n-3) generating DHA. In addition, this enzyme can also desaturate the n-6 substrate adrenic acid (ADA, 22:4n-6) to generate ω6-DPA (22:5n-6).

Although the predicted Δ4 desaturase shares similar structural characteristics with front-end desaturases, it is shown to be larger than known front-end desaturases (519 residues), displaying an additional 80 residues between the second and third conserved histidine motifs. It is not known if this size difference is responsible for the catalytic specificity of the enzyme. This enzyme shares only ~28–30% amino acid identity to other known front-end desaturases, with regions of homology located mainly in the cytochrome *b*5-like domains, and the histidine-box motifs.

The *fad4* (D4 saturase gene) gene has been expressed in *B. juncea*, to determine functionality in oilseed crop [69]. In transgenic *Brassica*, the leaves, stems and roots, rapidly took up the exogenously supplied substrate, and DHA was produced at 3–6% of total fatty acids. Thus this gene has potential for use in production of DHA-enriched oil.

4. Conclusion and future perspectives

Over the last few years, major advances have been made in the cloning and identification of fatty acid desaturase genes from an array of different organisms. This information has helped gain new insights into the PUFA biosynthetic machinery, and the physiological

functions of PUFAs. A plethora of information exists on the role of dietary PUFAs in the prevention and alleviation of chronic disease conditions. Current research is more focused on understanding the mechanisms regulating desaturase gene expression and activity, with the hope of gaining new insights into human diseases associated with lipid dysfunction.

The study of fatty acid desaturases has important biotechnological applications involving the engineering of new plant oils to meet the increasing demands of the chemical, pharmaceutical and nutraceutical industry. Advances in desaturase studies will impact the single-cell oil industry and the marine fish farming industry, all of which depend on PUFAs generated by microalgae and fungi.

From an academic standpoint, identification of novel desaturases will help further our understanding of the evolutionary relationships between organisms, and the evolution of PUFA metabolism in organisms. Although the overall scheme of PUFA biosynthesis appears to be common for most organisms, different organisms appear to have evolved distinct mechanisms of PUFA biosynthesis in adaptation to their environment. This is exemplified by the finding of a bi-functional $\Delta 5/\Delta 6$ desaturase in zebrafish, and the discovery of two distinct pathways for DHA synthesis that co-exist in organisms that produce copious amounts of DHA.

What remains to be carried out is the structural characterization of the purified membrane-bound desaturases. Work has been slow in this area due to technical difficulties involved in obtaining large quantities of purified membrane-bound proteins. Until then, molecular genetic approaches will be the resource used to gain insight into the structure–function relationship of these different desaturases.

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STRUCTURE, FUNCTION, AND DIETARY REGULATION OF Δ6, Δ5, AND Δ9 DESATURASES

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■ **Abstract** Fatty acid desaturases introduce a double bond in a specific position of long-chain fatty acids, and are conserved across kingdoms. Degree of unsaturation of fatty acids affects physical properties of membrane phospholipids and stored triglycerides. In addition, metabolites of polyunsaturated fatty acids are used as signaling molecules in many organisms. Three desaturases, Δ9, Δ6, and Δ5, are present in humans. Delta-9 catalyzes synthesis of monounsaturated fatty acids. Oleic acid, a main product of Δ9 desaturase, is the major fatty acid in mammalian adipose triglycerides, and is also used for phospholipid and cholesteryl ester synthesis. Delta-6 and Δ5 desaturases are required for the synthesis of highly unsaturated fatty acids (HUFAs), which are mainly esterified into phospholipids and contribute to maintaining membrane fluidity. While HUFAs may be required for cold tolerance in plants and fish, the primary role of HUFAs in mammals is cell signaling. Arachidonic acid is required as substrates for eicosanoid synthesis, while docosahexaenoic acid is required in visual and neuronal functions. Desaturases in mammals are regulated at the transcriptional level. Reflecting overlapping functions, three desaturases share a common mechanism of a feedback regulation to maintain products in membrane phospholipids. At the same time, regulation of Δ9 desaturase differs from Δ6 and Δ5 desaturases because its products are incorporated into more diverse lipid groups. Combinations of multiple transcription factors achieve this sophisticated differential regulation.

CONTENTS

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INTRODUCTION

Unsaturation of a fatty acid chain is a major determinant of the melting temperature of triglycerides (TGs) as well as the fluidity of biological membranes that are made of a bilayer of phospholipids (PLs). Thus, fatty acid desaturases that introduce a double bond into a long-chain fatty acid are conserved across kingdoms. In addition to this fundamental function of maintaining the physical property of PLs and TGs, another class of unsaturated fatty acids such as arachidonic acid (20:4 n-6) and docosahexaenoic acid (22:6 n-3) is essential for many physiological functions in animals including humans. Three desaturases are known in humans. Stearoyl CoA desaturases (SCDs, also called Δ9 desaturases) catalyze synthesis of monounsaturated fatty acids (MUFAs), whereas Δ6 desaturase (D6D) and Δ5 desaturase (D5D) are required for the synthesis of highly unsaturated fatty acids (HUFAs) (Figure 1). The primary focus of this review is mammalian D6D and D5D. Another mammalian desaturase, SCD, is also discussed in this review to present overlapping as well as distinct roles of these three desaturases. Desaturases in other species are also covered briefly to review the physiological roles of desaturases in a larger context.

SEQUENCE AND STRUCTURE OF DESATURASES

Classifications and Characteristic Features of Desaturases

Because unsaturated fatty acids are essential for maintaining cellular functions, free-living organisms possess fatty acid unsaturation machineries. In many prokaryotes, fatty acid unsaturation is achieved anaerobically by components of the fatty acid synthetic pathway (85). Another mechanism catalyzed by fatty acid desaturases introduces a double bond into fatty acids aerobically. Fatty acid desaturases are nonheme iron-containing enzymes that introduce a double bond between defined carbons of fatty acyl chains. Delta desaturases create a double bond at a fixed position counted from the carboxyl end of fatty acids, whereas omega desaturases act on a specific position counted from the methyl end of a fatty acid. This reaction requires molecular oxygen, NAD(P)H, an electron transport system (ferredoxin-NADPH reductase and ferredoxin, or cytochrome *b*5 reductase and cytochrome b_5), and a terminal desaturase.

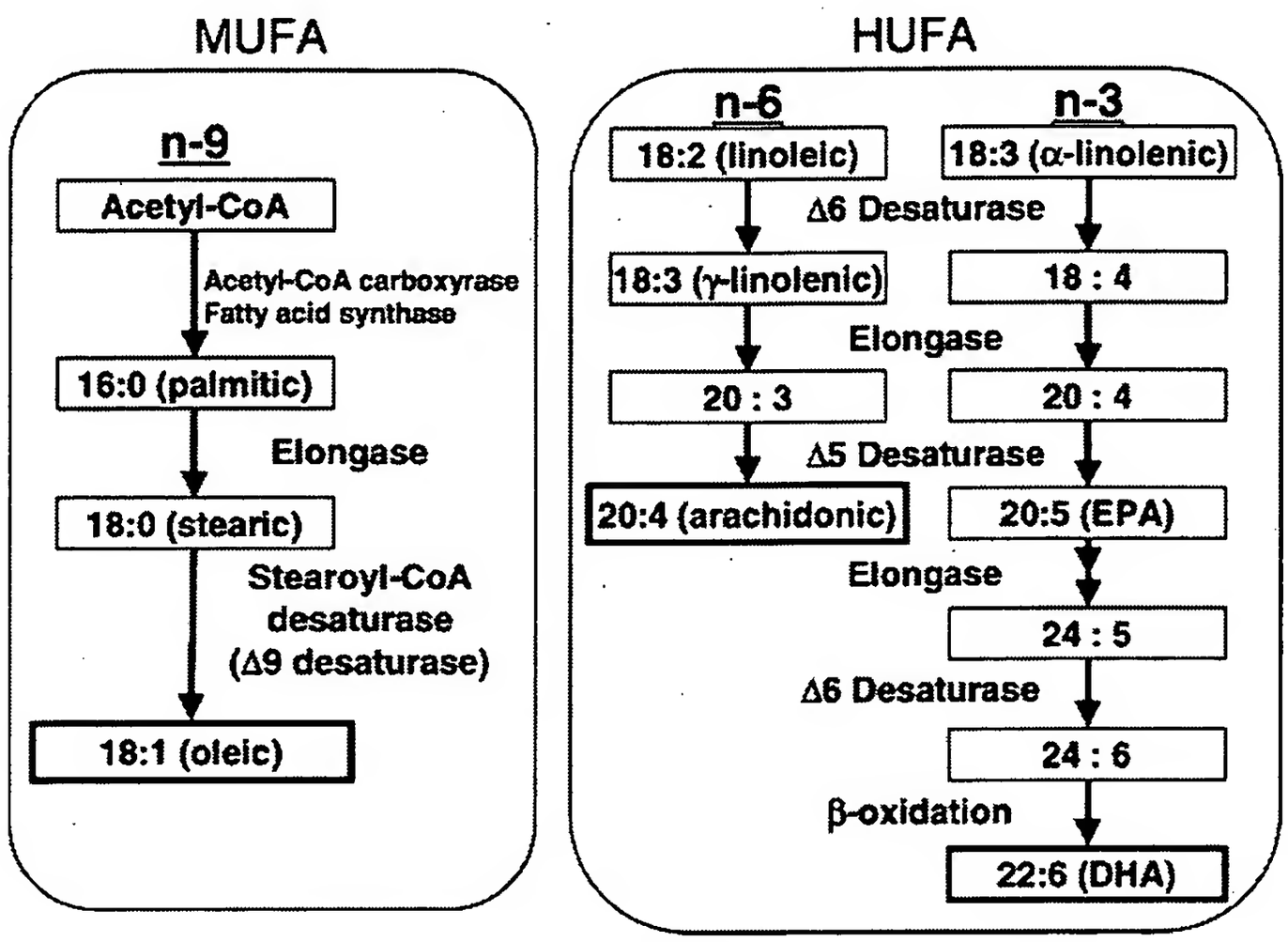

Figure 1 Synthesis of unsaturated fatty acids in mammals. MUFA, monounsaturated fatty acid; HUFA, highly unsaturated fatty acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

Desaturases can be classified into two groups: soluble desaturases and membrane-bound desaturases. Acyl-acyl carrier protein (ACP) desaturases are soluble desaturases, which are exclusively localized in the plant plastid. These enzymes require NADPH and oxygen, and are associated with an electron transport sequence comprising ferredoxin-NADPH reductase and ferredoxin (157). Acyl-ACP desaturases contain two atoms of iron and the two D/EXXH motifs of amino acid sequence involved in binding the di-iron complex (28, 157, 163).

Membrane-bound desaturases can be divided into two subgroups. One is the acyl-lipid desaturases. This group of enzymes is localized in the membranes of cyanobacterial thylakoid, plant endoplasmic reticulum (ER), and plastid. Acyl-lipid desaturases use either ferredoxin (in cyanobacteria and plant plastid) or cytochrome b_5 (in plant ER) as an electron donor (177). Ferredoxin, a soluble protein, acts as an electron donor for both acyl-ACP desaturases and acyl-lipid desaturases in plant plastid. Acyl-lipid desaturases in cyanobacteria and plant plastid can desaturate stearic (18:0) and oleic (18:1 n-9) acyl groups in monogalactosyl diacylglycerol (cyanobacteria and plant plastid) and in phosphatidylglycerol (plant plastid), whereas plant ER desaturases mostly use fatty acid in phosphatidylcholine (119, 177).

The other subgroup of membrane-bound desaturases is the acyl-coenzyme A (CoA) desaturases. These desaturases are present in ER membrane and use

fatty acyl-CoAs as substrates. Like ER-bound acyl-lipid desaturases, acyl-CoA desaturases require cytochrome b_5 as an electron donor (177). Acyl-CoA desaturases are present in animals including insects and nematodes as well as in fungi. All mammalian desaturases that have been identified are acyl-CoA desaturases.

The analysis of the predicted amino acid sequences of membrane-bound desaturases indicates that these enzymes contain two long hydrophobic domains that would be capable of spanning the membrane bilayer twice. The comparison of sequences has also revealed the existence of three regions of conserved His-box motifs that contain eight histidine residues: $HX_{3-4}H$, $HX_{2-3}HH$, and $H/QX_{2-3}HH$. These histidine residues are potential ligands of iron atoms and act at the catalytic center of desaturases. Acyl-ACP desaturases are structurally unrelated to membrane-bound desaturases.

Delta-9 Desaturases

STEAROYL-CoA DESATURASE Monounsaturated fatty acids (MUFAs) are synthesized from saturated fatty acids by Δ9 desaturases (D9Ds). These enzymes introduce the first *cis*-double bond at the 9, 10 position from the carboxyl end of fatty acids. Mammalian D9D, usually referred to as stearoyl-CoA desaturase (SCD), was first purified from rat liver (166). Subsequently, its gene was identified from the amino acid sequence (174, 175). SCD catalyzes the Δ9 desaturation of fatty acyl-CoA with 12 to 19 carbon chains (121). This reaction requires NADH, oxygen, and an electron transport sequence comprising NADH-cytochrome *b*5 reductase, cytochrome b_5, and SCD (22, 166). The sequence analysis indicates that this enzyme contains three regions of conserved His-box motifs. Site-directed mutagenesis study has shown that any of these conserved His residues are essential for the enzyme activity (158).

Four isoforms of stearoyl-CoA desaturases have been identified in mice (SCD-1, -2, -3, and -4), whereas only one SCD that is highly homologous to mouse SCD-1 is known in humans (100, 122). The four SCDs in mice exhibit tissue-specific expression. SCD-1 is expressed constitutively in adipose tissue and is markedly induced in liver in response to feeding with a high-carbohydrate diet (122). SCD-2 is expressed in the brain and harderian gland; SCD-3 is abundant in the harderian gland (122). SCD-4 was recently identified and is expressed exclusively in heart (100). Physiological roles and regulations of these SCD isoforms are unknown.

YEAST OLE1 Yeast *Saccharomyces cerevisiae OLE1* gene encodes the Δ9 fatty acyl-CoA desaturase. Ole1 protein catalyzes the desaturation of palmitic acid (16:0) and 18:0 to produce palmitoleic acid (16:1 n-7) and 18:1 n-9 (167, 168). This enzyme is ER membrane–bound and contains all the structural features of SCD, including two membrane-spanning domains and three His-box motifs. In addition, *OLE1* contains the cytochrome b_5 domain at its carboxyl terminal region (99). Mutation of the cytochrome b_5 domain resulted in the loss of complementation to the unsaturated fatty acid auxotrophy, which indicates that the cytochrome b_5 domain of Ole1 protein plays an essential role in the desaturase reaction (99).

DELTA-9 DESATURASES IN OTHER SPECIES The D9Ds in animals including insects, nematodes, and vertebrates share common features. For example, they are microsomal acyl-CoA desaturases and use NADH, cytochrome b5, and NADH-cytochrome *b*5 reductase. Similar to yeast, a D9D from a fungal strain, *Mortierella alpina* has a putative cytochrome b_5 domain at the C-terminal (151). In cyanobacteria *Anabaena variabilis* and *Synechocystis* sp. PCC 6803, acyl-lipid desaturases catalyze the Δ9 desaturation of 18:0 in the *sn-1* position of glycerolipids (150). In plants, soluble acyl-ACP desaturases catalyze this reaction (157, 177).

Delta-6 and Delta-5 Desaturases

DELTA-6 DESATURASES D6Ds are membrane-bound desaturases that catalyze the synthesis of polyunsaturated fatty acids (PUFAs). D6D was first cloned from *Synechocystis* using gain-of-function cloning (138). Subsequently, other D6Ds were cloned from *Borage officinalis* (155), *Caenorhabditis elegans* (114), humans (11), mice (11), and rats (1) using a sequence homologous to the *Synechocystis* D6D or other desaturases.

D6D is classified as a front-end desaturase because it introduces a double bond between the pre-existing double bond and the carboxyl (front) end of the fatty acid. Sequence analysis of deduced amino acid has shown that D6Ds contain the amino-terminal cytochrome b_5 domain carrying heme-binding motifs (Figure 2). The *Borage* D6D is an acyl-lipid desaturase that uses linoleate in phosphatidylcholine as a substrate (169), whereas D6Ds in other species are acyl-CoA desaturases. D6Ds also have two membrane-spanning domains and three His-box motifs that are characteristic of membrane-anchored desaturases. However, the first His residue of the third His-box is replaced with glutamine (QXXHH instead of HXXHH). A study using site-directed mutagenesis of borage D6D revealed that this glutamine residue is essential for D6D activity because mutations of glutamine to histidine as well as glutamine to isoleusine abolished the activity (154). Point mutation of the cytochrome b_5 domain revealed that this domain was also essential for the activity of D6Ds (35, 154).

DELTA-5 DESATURASES D5D, another front-end desaturase present in animals, catalyzes HUFA synthesis (Figure 1). After desaturation and elongation by D6D and elongase, respectively, D5Ds introduce another double bond at the Δ5 position of 20-carbon fatty acids 20:3 n-6 and 20:4 n-3 (Figure 1). D5D genes have been cloned from several animals including humans (10), rats (200), and *C. elegans* (186). The human D5D gene encodes 444 amino acids (the same number as the human D6D) and possesses 61% amino acid identity and 75% similarity to the human D6D. The predicted amino acid sequence of D5D contains all of the structural characteristics present in D6Ds (10).

Recently, Hastings et al. cloned and characterized a zebrafish (*Danio retio*) desaturase that is homologous to both D6D and D5D (38). The deduced amino acid sequence of this enzyme shared 64% and 58% identity with human D6D and

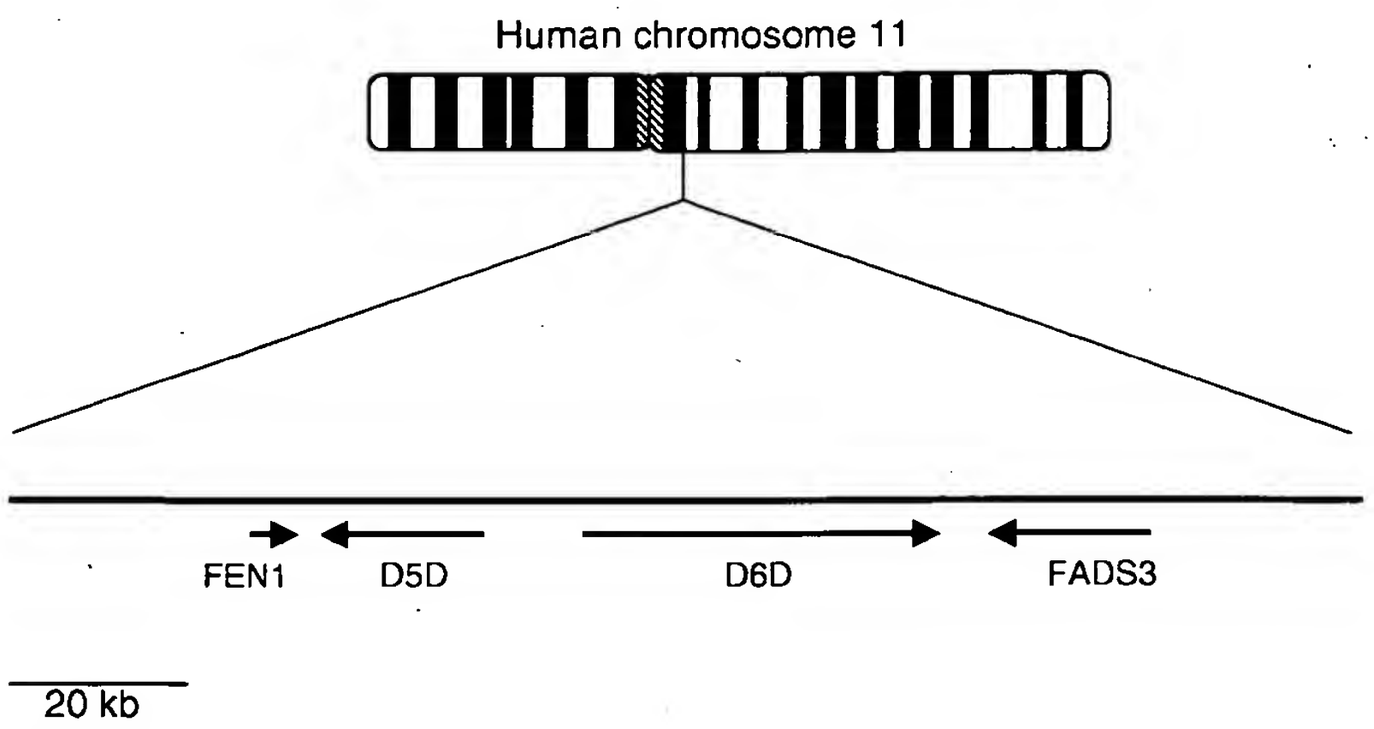

Figure 2 Localization of the human desaturase genes. D5D, Δ5 desaturase; D6D, Δ6 desaturase; FADS3, fatty acid desaturase 3; FEN1, flap-end nuclease 1.

D5D, respectively. This enzyme was able to convert not only linoleic (18:2 n-6) and α-linolenic (18:3 n-3) acids to 18:3 n-6 and 18:4 n-3, but also produced arachidonic (20:4 n-6) and eicosapentaenoic (20:5 n-3) acids when the enzyme was expressed in yeast (38). This result indicates that the zebrafish desaturase possesses both D6D and D5D activities. The existence of Δ5/Δ6 bifunctional desaturase implies that Δ6 and Δ5 desaturases are evolved from common ancestors.

LOCALIZATION OF HUMAN *D5D*, *D6D*, AND *FADS3* GENES IN HUMAN CHROMOSOME

The computer search of the NCBI database (human genome resources) has shown that *D5D* and *D6D* genes are localized in chromosome 11 (11q12–q13.1) as a cluster (Figure 2). Both *D5D* and *D6D* genes consist of 12 exons and 11 introns spanning the 17.2 kb and 39.1 kb regions, respectively. Interestingly, *D5D* and *D6D* genes are oriented head-to-head, and exon 1 of both genes is separated by the 11 kb region. The proximity of the promoters suggests the possibility that transcription of the *D5D* and *D6D* genes may be coordinately controlled by common regulatory sequences within the 11 kb region. The third putative desaturase gene (fatty acid desaturase 3, *FADS3*) also has 12 exons and 11 introns. *FADS3* is located in the 6.0 kb telomeric side from the *D6D* gene and is oriented in a tail-to-tail manner (Figure 2). *D5D*, *D6D*, and *FADS3* form a cluster within the 100 kb region of human chromosome and have the same exon/intron organization, which suggests that these desaturases have arisen evolutionarily from gene duplication.

The mouse homologues of *D5D*, *D6D*, and *FADS3* are located in mouse chromosome 19 as a cluster. A sequence analysis of a BAC clone (accession no. AC026761) and a mouse genomic database search shows that the orientation and exon/intron organization of these mouse desaturase genes are similar to that of human homologues.

The human *FADS3* gene has an open reading frame of 1338 bp that encodes 445 amino acids, while the mouse *FADS3* gene possesses a 1350 bp open reading frame for 449 amino acids. The predicted amino acid sequence contains all of the conserved structural features of *D6D*. Alignment of the putative amino acid sequence of human *FADS3* with *D5D* and *D6D* demonstrated that *FADS3* had a high degree of identity (52% with *D5D*, 62% with *D6D*) (87). However, the function of this gene product is unknown. A search of the human expressed sequence tag databases revealed that *FADS3* mRNA was expressed in brain, placenta, ovary, B-cell, and skin as well as in fetal brain.

Omega-3 Desaturases

Omega-3 (n-3) desaturases catalyze a reaction that introduces a double bond between three and four carbons from the methyl end of fatty acids. Amino acid sequences of ω3 desaturases contain three conserved His-box motifs and two membrane-spanning domains that are characteristic of membrane-bound desaturases (177). Like other acyl-lipid desaturases, ω3 desaturases also require ferredoxin (plastid) or cytochrome b_5 (plant ER) (177). Vertebrates do not have ω3 desaturases and consequently must obtain n-3 PUFAs from their diet, but a wide range of organisms other than vertebrates can synthesize ω3 PUFA. The *desB* gene of the cyanobacterium *Synechocystis* sp. PCC 6803 encodes an ω3 desaturase (149). This desaturase is localized in plastid and catalyzes the introduction of a double bond into 18:2 n-6 esterified in the *sn-1* position of glycerolipids (149). The higher plant *Arabidopsis thaliana* has three ω3 desaturases: FAD3, FAD7, and FAD8. In ER, FAD3 introduces a double bond in the ω3 position of 18:2 n-6 to produce 18:3 n-3 using phosphatidylcholine as the major substrate (7). FAD7 and FAD8 are localized in chloroplasts and desaturate glycolipid-bound 16:2 n-6 and 18:2 n-6 to 16:3 n-3 and 18:3 n-3, respectively (49, 95). In nematode *C. elegans*, *fat-1* encodes an ω3 desaturase (165). Both plant and nematode ω3-desaturases have preference to the substrates that contain an n-6 double bond (96, 141).

FUNCTION OF DESATURASES

Physiological Roles of Stearoyl CoA Desaturases

As shown in Figure 1, mammals have all enzymes for the synthesis of MUFAs from acetyl CoA. SCD catalyzes the last step of this synthesis. The main product, oleic acid (18:1 n-9), is ubiquitously present in all tissues. In mammals, adipose TGs mainly consist of long-chain fatty acids with 16 and 18 carbons. Certain amounts of unsaturated fatty acids (18:1 n-9 and 18:2 n-6) are required to maintain physical property at the body temperature of mammals. In humans, 18:1 n-9 is the major species in adipose TGs, comprising nearly half of total fatty acids (135). Therefore, SCD is a vital component for de novo lipogenesis to store excess energy as a TG form. Indeed, disruption of the *SCD-1* gene in mice profoundly affects

energy metabolism and makes the animal resistant to developing obesity (14, 122, 123, 137).

The significant amount of 18:1 n-9 present in PLs in various tissues and serum in mammals (111, 133, 135) contributes to maintenance of biological membrane fluidity. As shown later in this section, yeast has only one desaturase (Ole1, a Δ9 desaturase), which is sufficient to maintain the physical property of membranes in this organism. In contrast to HUFAs, 18:1 n-9 in membrane PLs does not have a role in cell signaling.

SCD is also required for cholesteryl ester (CE) synthesis in liver (101), and is induced by dietary cholesterol (63, 72). Studies suggest that SCD plays a vital role in cholesterol metabolism by providing a substrate for CE synthesis to temporarily store excess cholesterol in liver (63, 72, 101). Another function of SCD was discovered in SCD-1-null mice, which showed defects in lipid synthesis and secretion from skin and eyelid (102). Dietary supplement of 18:1 n-9 did not correct this abnormality, indicating requirement of endogenous synthesis of MUFAs in these tissues. The involvement of SCD in these multiple metabolic pathways requires complex regulation of this gene by various nutrients as discussed in the next section.

Functions of Δ6 and Δ5 Desaturases

SYNTHESIS OF HUFAs IN MAMMALS HUFAs, such as arachidonic acid (20:4 n-6) and docosahexaenoic acid (22:6 n-3) are required for various physiological functions for mammals including humans. Mammals are unable to synthesize HUFAs from acetyl CoA because neither ω3 desaturase nor Δ12 desaturase is present in mammals. Thus, two PUFAs, 18:2 n-6 and 18:3 n-3, termed essential fatty acids (EFAs), must be supplied from diets. Mammals are able to synthesize HUFAs from these precursor PUFAs (164). Two desaturases, D6D and D5D, are required for the synthesis of HUFAs (Figure 1). Both D6D and D5D are widely expressed in human tissues, with the highest levels in liver (10, 11).

Animal studies showed that the precursors 18:2 n-6 and 18:3 n-3 are readily stored in adipose TGs, which work as a reservoir of EFAs (81, 92). In humans, fatty acid composition in adipose tissue reflects dietary fatty acids (30). In contrast to MUFAs and precursor PUFAs, HUFAs are poor substrates for TG synthesis, and mainly incorporated into PLs (199), contributing to maintenance of membrane fluidity. However, maintaining membrane fluidity is not the main function of HUFAs in mammals. HUFAs are required for many other functions, such as eicosanoid signaling (29), pinocytosis (156), ion channel modulation (57), and regulation of gene expression (12).

ARACHIDONIC ACID SYNTHESIS AND EICOSANOID SIGNALING Arachidonic acid (20:4 n-6) is one of two major HUFAs synthesized by the D6D/D5D pathway (Figure 1). In many tissues and cell types, 20:4 n-6 is esterified to the *sn-2* position of membrane PLs, and is used for the eicosanoid-mediated signaling to

perform specialized cell functions (199). Arachidonic acid esterified in PLs is a storage form and must be hydrolyzed first to be converted to eicosanoids. Upon stimulation or cell injury, free arachidonic acid is released from PLs by phospholipases, and then is enzymatically converted to eicosanoids (29, 153). Eicosanoids work as autocrine/paracrine hormones and mediate a variety of localized reactions, such as inflammation (29), hemostasis (153), and protection of digestive tract epithelium (130, 183). A human case of D6D deficiency showed severe food intolerance and growth retardation. These symptoms were reversed by arachidonic acid supplementation (191), which indicates the essential nature of eicosanoids in the protection of digestive tract mucosa in humans.

DELTA-6 DESATURASE IN DOCOSAHEXAENOIC ACID SYNTHESIS Docosahexaenoic acid (22:6 n-3) is another important product of the D6D/D5D pathway. The synthesis of 22:6 n-3 shares a pathway with 20:4 n-6 up to the 20:5 n-3 step. D6D is also required for the synthesis of 22:6 n-3 from 20:5 n-3 (Figure 1). The synthetic pathway of 22:6 n-3 from 20:5 n-3 was first proposed by Voss et al. (180) based on the following observations: (*a*) there was no detectable Δ4 desaturase activity in mammals, and (*b*) 24:5 n-3 was desaturated to 24:6 n-3 and then was readily retroconverted to 22:6 n-3 in rat liver. Recent studies suggest that the final desaturation in 22:6 n-3 synthesis may be catalyzed by the same D6D that catalyzes the first step. Fibroblast from a human case of D6D deficiency was unable to desaturate either 18:2 n-6 or 24:5 n-3 (191). Another study showed that rat D6D was able to desaturate both 18:3 n-3 and 24:5 n-3 at the Δ6 position (18). No D6D isozyme specific to 24-carbon fatty acid is known in any species at this time.

Voss et al. also proposed that peroxisome might be the site of β-oxidation for the last step of 22:6 n-3 synthesis (180) because tissue 22:6 n-3 was decreased in peroxisomal disorders (88). This hypothesis is supported by recent studies. The peroxisomal β-oxidation is carried out by three enzymes: acyl CoA oxidase, bifunctional protein, and 3-ketoacyl CoA thiolase. Each of these enzymes has isozymes (139). Studies with human skin fibroblast showed that a deficiency of either straight-chain acyl CoA oxidase or D-bifunctional protein greatly reduced the 22:6 n-3 synthesis, but it did not completely abolish it (23, 170). An in vivo study using straight-chain acyl CoA oxidase-null mice showed the same results as the studies with fibroblasts (51). These studies have demonstrated a critical role of peroxisomal β-oxidation in 22:6 n-3 synthesis, and suggest that straight-chain acyl CoA oxidase and D-bifunctional protein play the major role in the 22:6 n-3 synthesis, but other peroxisomal isozymes can catalyze the reaction in a lesser degree.

Peroxisomes play the major role in the oxidation of very long chain fatty acids (20 or more carbons) (140). Consistent with this function of peroxisome, dietary fish oil rich in 20:5 n-3 and 22:6 n-3 induces peroxisomal oxidation enzymes (2, 116). Therefore, peroxisomes are likely to be involved in both synthesis and oxidation of 22:6 n-3. Although it is currently unknown how synthesis and degradation of 22:6 n-3 are carried out and regulated in the same organelle, it is possible that

22:6 n-3 required for cellular functions is rapidly incorporated into PLs or transported out of peroxisomes, whereas excess 22:6 n-3 remains in peroxisomes and undergoes further degradation.

FUNCTIONS OF 22:6 n-3 Docosahexaenoic acid is abundant in excitable membranes in retina and brain. In particular, 22:6 n-3 is high in PLs in the rod outer segment of retina and in synaptic vesicles and plasma membrane of neurons (152). Docosahexaenoic acid is essential for the function of retina. Studies with primates and rodents showed that a deficiency of n-3 PUFAs causes impairment in visual function (117, 118). In humans, inclusion of 22:6 n-3 in formula accelerated the development of visual functions in preterm infants (40, 126). However, the mechanism of 22:6 n-3 functions in retina is not well understood. Chen et al. suggested that 22:6 n-3 in retina might be involved in shuttling 11-*cis*-retinal to photoreceptors (9), whereas Salem et al. proposed that 22:6 n-3 in PLs increases efficiency of G-protein-mediated signal transduction of rhodopsin (152).

The role of 22:6 n-3 in brain function is less clear. Recent studies reported impairment of spatial task (105) and olfactory-based learning (33) by deprivation of n-3 fat. In humans, patients with dementia including Alzheimer's disease showed low 22:6 n-3 in plasma lipids (15). Also, improvement of neuronal cell survival by n-3 fatty acids suggests that 22:6 n-3 is tied to differentiated functions of neurons (31, 69). The mechanism of 22:6 n-3 in brain functions is unknown. Salem et al. suggested 22:6 n-3 plays a similar role in both retina and brain (152). A recent study has shown an involvement of 20:4 n-6 in neurotransmitter recycling (156), a finding that implies 22:6 n-3 may also have the same function in neurons containing high 22:6 n-3.

OTHER FUNCTIONS OF HUFAs HUFAs are involved in cellular functions in addition to those described above. Classical studies of the essential fatty acid deficiency in rodents demonstrated main symptoms of dry skin, dermatitis, and massive water loss through skin (42, 132). These symptoms were reversed by dietary n-6 or n-3 PUFAs, although 18:3 n-3 is less effective than 18:2 n-6 (42). Subsequent studies have shown that 18:2 n-6 is required in skin ceramides to prevent water loss (50, 172). However, in a human case of D6D defect, severe abnormalities were also reported in skin, hair, and nail, which indicated that desaturation of substrate PUFAs by D6D is required for these functions. Moreover, dietary supplementation of HUFAs did not reverse these symptoms completely (191), highlighting the importance of the endogenous D6D pathway for skin functions. Taken together, these studies suggest that 18:2 n-6 is required not only for ceramide synthesis but also for HUFA synthesis, although the function of HUFAs in skin has yet to be elucidated.

Another potentially important function of HUFAs is prevention of cardiac arrhythmia during reperfusion after ischemia (71). In heart, HUFAs are released from membrane PLs under hypoxia. These nonesterified HUFAs facilitate synchronizing the contraction of cardiac myocytes upon reperfusion by increasing the

recovery time of ion channels. This effect is more pronounced in n-3 HUFAs (20:5 n-3 and 22:6 n-3) than in 20:4 n-6, and is the likely mechanism by which dietary fish oil could reduce deaths from myocardial infarction. Another study suggests that by slowing the ion channel activity, n-3 HUFAs may also alleviate the symptoms of convulsive seizures, which are characterized by uncontrolled firings of neurons in the central nervous system (181).

Neurotransmitters are taken up and stored for reuse in very small vesicles via pinocytosis. A study by Schmidt et al. found that endophilin, an essential component in neurotransmitter recycling, is a lysophosphatidic acid acyl transferase (156). Their study suggests that rapid hydrolysis of 20:4 n-6 by phospholipase A2 and reacylation by lysophospholipid acyl transferases change the membrane curvature, and thus play a critical role in pinocytosis in neurons. Docosahexaenoic acid in brain may also serve as a substrate of phospholipases and acyl transferases during pinocytosis because as mentioned previously 22:6 n-3 is highly enriched in the PLs of synaptic vesicles and plasma membrane of neurons (152). It is possible that this postulated function of HUFAs is also used for endocytosis and exocytosis in other tissues such as for insulin secretion in pancreas (53) and for nutrient transport through amniotic fluid during pregnancy (75).

It has long been known that dietary PUFAs suppress lipogenesis in liver (13). In past decades, it became clear that PUFAs and their metabolites exert this effect by regulating gene expression (12, 55). Recent developments further revealed that HUFAs regulate various transcription factors, such as PPAR, liver-X receptor (LXR), HNF4, and SREBP (54). Regulation of desaturase genes by PUFAs is discussed in the next section.

Desaturase Pathway and Implications for Dietary Requirement of PUFAs

IMPORTANCE OF BALANCED SUPPLY OF n-6 AND n-3 PUFAs Cloning of mammalian *D6D* and *D5D* genes demonstrated that the same enzymes catalyze the synthesis of both n-6 and n-3 HUFAs as shown in Figure 1 (10, 11). Moreover, as discussed in detail in the next section, HUFA synthesis is under strong feedback regulation. This indicates that a balanced supply of dietary n-3 and n-6 fatty acids is important to meet the requirement of both n-3 and n-6 HUFAs. As discussed in the Synthesis of HUFA in Mammals section, both 18:2 n-2 and 18:3 n-3 from diets can be stored in large quantity as adipose TGs (30, 81, 92). However, analyses of fatty acid composition showed very low 18:3 n-3 in adipose TGs in the U.S. population (30, 135), raising concerns that the U.S. dietary supply of 18:3 n-3 may be marginal or deficient. Symptomatic n-3 deficiency has not been identified in humans except for a patient who received lipid emulsion lacking n-3 fatty acid intravenously for a prolonged period (43, 45). Animal studies show that depletion of brain n-3 HUFA is a slow process (117, 118, 152). Therefore, the consequence of marginal n-3 deficiency in humans may take many years to manifest, and could be masked with the loss of brain function by aging.

POPULATION GROUPS WITH POSSIBLE INSUFFICIENT ENDOGENOUS HUFA SYNTHESIS HUFAs in serum PLs in healthy adults are maintained in a narrow range despite differences in intake of precursor PUFAs (134). Animal studies have shown that tissue 20:4 n-6 stays constant over a wide range of dietary 18:2 n-6 (103, 136). D6D and D5D are fully induced only in EFA-deficient conditions, and are suppressed when adequate precursor PUFAs are supplied from the diet (10, 11), indicating that the capacity of endogenous synthetic pathway is sufficient to meet the requirement of HUFAs in healthy adults.

However, dietary HUFA supplementation may become necessary for certain populations whose endogenous HUFA synthesis is insufficient. Studies indicate that supplementation of n-6 and n-3 HUFAs in preterm infants improves growth and cognitive development (40, 126). Indeed, a search of expressed sequence tag databases reveals the expression of the *D6D* mRNA in human fetus, infant, amnion, uterus, and breast, suggesting maternal contribution of the HUFA supply as well as active HUFA synthesis by fetuses and infants. The elderly is another group that might have insufficient endogenous synthesis of HUFAs. Epidemiological data suggest that consumption of fish oil may be beneficial in preventing dementia (106). As mentioned previously, a chronic marginal deficiency of 18:3 n-3 may be exacerbating the decline of 22:6 n-3 in elderly. Other disease states in which tissue HUFA content and/or synthesis is decreased include diabetes (44, 146), insulin resistance (4), peroxisome disorders (89, 107), and alcoholism (111–113). Although supplementation of dietary HUFA may benefit patients with these diseases, decreased HUFAs are unlikely the central pathology of these disorders.

DIETARY HUFAs AS FUNCTIONAL FOODS Dietary HUFAs also have a property of functional foods because HUFAs exert additional health benefits and therapeutic effects when supplied from diet beyond requirement. When supplied from diets, fish oil rich in 20:5 n-3 and 22:6 n-3 shows a hypotriglyceridemic effect (64) by suppressing secretion of very low-density lipoproteins (25). Dietary n-3 HUFAs also exhibit an anti-inflammatory effect (20) because of a counteracting property of n-3 HUFAs against the formation and actions of 20:4 n-6-derived eicosanoids (67). These studies suggest that combining dietary n-3 HUFAs with a drug therapy may reduce the dose of the drugs required to treat these disorders. However, care must be taken in the use of HUFAs as a functional food because an excessive supplementation of one HUFA (n-3 or n-6) may cause a deficiency in the other. Supplying HUFAs from diets means bypassing the regulation of the endogenous synthetic pathway, and poses potential problems. First, dietary HUFAs markedly change the n-6/n-3 HUFA ratio in PLs because n-6 and n-3 HUFAs compete for the esterification to PLs (189). Second, one group of dietary HUFAs would shut down the synthetic pathway shared by both n-3 and n-6 fatty acids (173), exacerbating the imbalance of n-6/n-3 HUFAs.

Physiological Roles of Desaturases in Other Organisms

UNICELLULAR ORGANISMS Unsaturated fatty acids in PLs increase the membrane fluidity necessary to maintain the proper function of biological membranes. In

particular, desaturation plays an important role in adaptation to a cold temperature in unicellular organisms. In cyanobacteria *Synechocystis* sp. PCC 6803, the growth rate of Fad12 (a Δ12 desaturase) mutant was much lower than the wild type at 22°C but the same at 34°C (182). Also, *desB*, ω3 desaturase in *Synechocystis* sp. PCC 6803, is induced by cold temperature (149). *Escherichia coli* mutants lacking the enzymes in the biosynthesis of unsaturated fatty acid showed auxotrophy for unsaturated fatty acids (16). Ole1, a D9D, is the only desaturase in *S. cerevisiae*, and is required for growth in all temperatures. Mutation of ole1 turns *S. cerevisiae* to auxotroph for oleate (144). *OLE1* mRNA is also increased by cold temperatures, resulting in increased MUFAs in membrane PLs (110).

PLANTS In higher plants, the cold tolerance is closely correlated with the level of unsaturated fatty acids in phosphatidylglycerol from chloroplast membrane, especially in the *sn-1* position (119). Plants synthesize 18:2 n-6 and 18:3 n-3 from saturated fatty acid 18:0 that are catalyzed by soluble acyl-ACP D9D and membrane-bound Δ12 and ω3 desaturases (119, 177). A *fad2*, *fad6* double mutant of *Arabidopsis thaliana*, deficient with Δ12 desaturase activity, was unable to synthesize PUFAs and showed reduced chlorophyll content and photosynthesis (94). Interestingly, tolerance to high temperature was improved in the transgenic tobacco (knockdown) and *Arabidopsis* mutant, both of which had reduced activity of chloroplast ω3 desaturase (109). The amount of 18:3 n-3 and 16:3 n-3 in chloroplast was lower in these mutants than wild type, whereas 18:2 n-6 and 16:2 n-6 were higher. These results suggest that fatty acid unsaturation determines the temperature range for an optimal chloroplast function in plants.

In addition, PUFAs are used as precursors for the synthesis of oxylipids, which are important signaling molecules for plants. For example, jasmonic acid is synthesized from 18:3 n-3 and participates in various plant physiological functions. A *fad3*, *fad7*, *fad8* triple mutant of *Arabidopsis* that lacked all ω3 desaturases was unable to induce jasmonic acid–mediated defense genes upon microbial infection (145, 179). Pollen development was also impaired in the *fad3*, *fad7*, *fad8* triple mutant (93).

ANIMALS In poikilotherms such as fish, unsaturated lipids in membrane are required to adapt to cold temperature. In carp (*Cyprinus carpio*), unsaturation in phospholipid increased at a cold temperatures (178). The D9D activity in carp liver increased without increasing the protein amount shortly after cooling; this was followed by an increase in the amount of transcript from two days after the treatment (176). Eicosanoids are important signaling molecules in fish as well as in mammals. Cyclooxygenese catalyzes the first step of conversion of 20:4 n-6 to prostaglandins (29). Zebrafish have both cyclooxygenase-1 and -2. In zebrafish, thrombocyte aggregation was prevented and bleeding time was prolonged by a cyclooxygenase-1 inhibitor, indomethacin (34). Furthermore, embryo development was impaired by knockdown of the cyclooxygenase-1 gene in zebrafish (34).

A nematode *Caenorhabditis elegans* is capable of synthesizing all necessary PUFAs because *C. elegans* expresses the full range of desaturases such as ω3

desaturase (FAT-1), Δ12 desaturase (FAT-2), D5D (FAT-4), D6D (FAT-3), and D9D (FAT-5, -6, and -7) (97, 114, 131, 165, 187). When *C. elegans* was cultured in a cold temperature, the proportion of PUFAs in PLs increased (172a). These changes in the PLs of *C. elegans* suggest a mechanism of adaptation to a cold temperature. Furthermore, both *fat-2* and *fat-3* mutants showed slow growth rate, sluggish movement, and a reduced brood size (188). The *fat-2* mutant *C. elegans* was deficient in almost all PUFAs and the *fat-3* mutant lacked 20-carbon PUFAs. These results suggest that PUFAs play an important role in the cell growth and neurological development of *C. elegans* (188).

Two D9Ds (Desat1 and Desat2) are the only desaturases identified in the *Drosophila* genome (190). Desat1 introduces the double bond in 16:0, whereas Desat2 prefers myristic acid (14:0) as a substrate. Insects such as the fruit fly *Drosophila* use unsaturated fatty acid to produce cuticular pheromones in addition to the synthesis of membrane PLs (17).

DIETARY REGULATION OF DESATURASES

Feedback Regulation of Desaturases by Dietary PUFAs in Liver

Mammalian cells require specific amounts of unsaturated fatty acids in PLs for the physical property of membrane and for differentiated cellular functions. In liver, unsaturated fatty acids are also required for synthesis of TGs and CEs. Mammalian desaturases are primarily regulated by induction of the enzyme (11, 120, 173). Promoter activities of desaturases are regulated by multiple transcription factors in liver to meet these various requirements of unsaturated fatty acids. Complex transcriptional regulation by combinations of multiple transcription factors is a mechanism by which higher organisms can achieve diverse functions without increasing the number of genes (77). Thus, regulatory mechanisms of desaturases present fascinating examples of sophisticated transcriptional regulations.

PUFAs are the main dietary component that regulates all three desaturases. Both D6D and D5D are suppressed by dietary PUFAs (10, 11, 129). PUFAs also suppress SCD (41, 124). The suppression of SCD by dietary fat is unique to n-6 and n-3 PUFAs. A product of SCD, 18:1 n-9, is less effective in vivo (41), and has no effect in cells in suppressing SCD expression (3). Two transcription factors, sterol regulatory element binding protein-1c (SREBP-1c) and peroxisome proliferator activated receptor-α (PPARα), play a key role in the regulation of desaturases by PUFAs.

ROLE OF SREBP-1c IN FEEDBACK REGULATION OF DESATURASES BY PUFAs SREBPs are transcription factors of the basic helix loop–helix leucine zipper (bHLHLZ) family, and were initially found as the factors that bind sterol regulatory element (SRE) in the promoter of the *low-density lipoprotein receptor* gene (5). SREBPs have two isoforms, SREBP-1 and SREBP-2, which are transcribed from different genes (48, 196). SREBP-1 has two subforms, SREBP-1a and SREBP-1c, which

are encoded from the same gene by alternative promoter usage (162). SREBP-2 mainly activates the transcription of genes involved with cholesterol synthesis and metabolism, whereas SREBP-1c targets genes for fatty acid synthesis, and SREBP-1a induces both (6, 47). The expression of SREBP-1a is high in dividing cells such as cell lines, whereas SREBP-1c is the major species in many differentiated cells, including hepatocytes (162). SREBPs are synthesized as a larger precursor protein that is anchored to the ER membrane. After proteolytic cleavage, the amino terminal domain migrates to a nucleus and activates target genes (6).

In liver, SREBP-1c activates entire genes of fatty acid synthesis, including all three desaturases (47, 91). SRE has been identified in prompters of many genes, including acetyl CoA synthase (82), acetyl CoA carboxylase (83), fatty acid synthase (84), SCD (171), D6D (115), elongase (104), and S14 (90).

Importantly, SREBP-1c also mediates the PUFA inhibition of D6D (115) and SCD (171, 184) as well as genes involved with fatty acid synthesis, such as fatty acid synthase (193) and S14 (90). PUFAs suppress the target gene transcription by reducing the active form of SREBP-1c (37, 90, 193, 194, 197). More than one mechanism is involved in this process. First, dietary PUFAs reduce nuclear form SREBP-1c in rats (193), and HEK293 cells (37), whereas dietary triolein (18:1 n-9) has no effect (193). Second, PUFAs reduce the stability of *SREBP-1c* mRNA (194). In addition, unsaturated fatty acids inhibit LXR-mediated activation of SREBP-1c by acting as antagonistic ligands in cell lines (127, 197). However, this transcriptional suppression of SREBP-1c by PUFAs is yet to be demonstrated in vivo. The mechanisms by which PUFAs reduce the SREBP-1c processing and the *SREBP-1c* mRNA stability are currently unknown. In *Drosophila* cells, phosphatidylethanolamine suppresses maturation of SREBP (19). In rats, membrane phosphatidylethanolamine was inversely correlated with D6D activity (160). Thus, PUFAs might exert suppression of SREBP-1c processing by changing PL composition in mammals as well.

The target genes of SREBP-1c are not limited to HUFA synthesis; they also include de novo MUFA synthesis, although only PUFAs, not MUFAs, suppress the activity of SREBP-1c. This broader range of targets implies that one physiological function of SREBP-1c is to maintain total unsaturated fatty acids in PLs, and possibly in TGs and CEs as well. Although both substrates and products of the D6D/D5D pathway suppress SREBP-1c processing and mRNA expression, HUFAs are more potent than precursor PUFA, 18:2 n-6 (90, 193). It is currently unknown whether conversion of precursors to HUFAs is required to exert suppression of SREBP-1c.

ROLE OF PPARα IN DESATURASE REGULATION PPARα is a transcription factor of the nuclear receptor family. Like other members of the family, PPARα has a hydrophobic ligand-binding pocket and DNA-binding domain. Binding of a ligand causes a conformational change to PPARα, which then forms a heterodimer with retinoid X receptor and activates transcription of target genes by binding peroxisome proliferator response element (PPRE) located in promoter regions of the

targets (8). Hypolipidemic compounds called peroxisome proliferators such as fibrates and Wy14643 induce fatty acid oxidation enzymes by acting as ligands of PPARα (27), and in rodents, cause peroxisome proliferation in liver (139). Nonesterified long-chain fatty acids are considered endogenous ligands of PPARα because a variety of long-chain fatty acids bind and activate PPARα (27, 65).

Target genes of PPARα include mitochondrial fatty acid oxidative enzymes (36), mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (147), cytochrome P450 (CYP) 4A (66), straight-chain acyl CoA oxidase, and peroxisomal L-bifunctional protein (86). Induction of fatty acid oxidation and ketogenesis are impaired in PPARα-null mice during starvation, resulting in hypothermia and hypoglycemia, whereas these animals grow normally when food is freely accessible (62, 70, 74). These studies demonstrated that PPARα plays a critical role in the metabolic adaptation to starvation by inducing genes for fatty acid oxidation.

Kawashima et al. first reported that fibrates increase the activity of D6D and SCD in rats (59, 60). Wy14643 strongly induces the *D6D* mRNA in rat liver (39). Nuclear run-on assay showed that transcriptional activation accounts for the induction of *D6D* mRNA by Wy14643 (173). Cell culture studies detected the presence of PPRE in the promoters of *SCD* (98) and *D6D* genes (173). The induction of desaturases by both PPARα and SREBP-1c is paradoxical because except for desaturases, these two transcription factors induce mutually exclusive sets of genes. PPARα in general induces genes of fatty acid oxidation, whereas SREBP-1 induces genes of fatty acid synthesis.

Peroxisome proliferators may induce desaturases in rodents partly by indirect mechanisms. First, administration of peroxisome proliferators is likely to increase the degradation of unsaturated fatty acids by inducing enzymes for fatty acid oxidation in both peroxisomes and mitochondria (139). Second, the requirement of unsaturated fatty acids for membrane PLs would be increased by administration of peroxisome proliferators, which induce proliferation of peroxisomes and enlargement of liver in rodents (60, 139). These changes would increase the demand of unsaturated fatty acids, resulting in induction of desaturases. Indeed, despite a strong induction of HUFA synthesis by peroxisome proliferators, little change was observed in HUFA composition in PLs (39, 60). Moreover, contrary to the rapid induction of PPARα-responsive genes, for fatty acid oxidation, such as *acyl CoA oxidase*, *carnitine palmitoyl transferase-1*, L-*bifunctional protein*, and *CYP4A*, the *D6D* and *D5D* mRNA took longer to reach maximum induction (39). The *SCD-1* gene also showed a delayed induction by clofibrate in mouse liver (98). These results indicate that indirect mechanisms contribute to the strong induction of desaturases by peroxisome proliferators in rodent liver.

In addition to potential indirect mechanisms, recently obtained data indicates that PPARα also directly activates the *D6D* gene and plays a crucial role in the feedback regulation of HUFA synthesis. The *D6D* mRNA was not induced in PPARα-null mice fed EFA-deficient diets, although nuclear SREBP-1c was elevated in both PPARα-null and wild-type mice (79). This demonstrated that SREBP-1c alone is not sufficient, and PPARα also is required to mediate the feedback

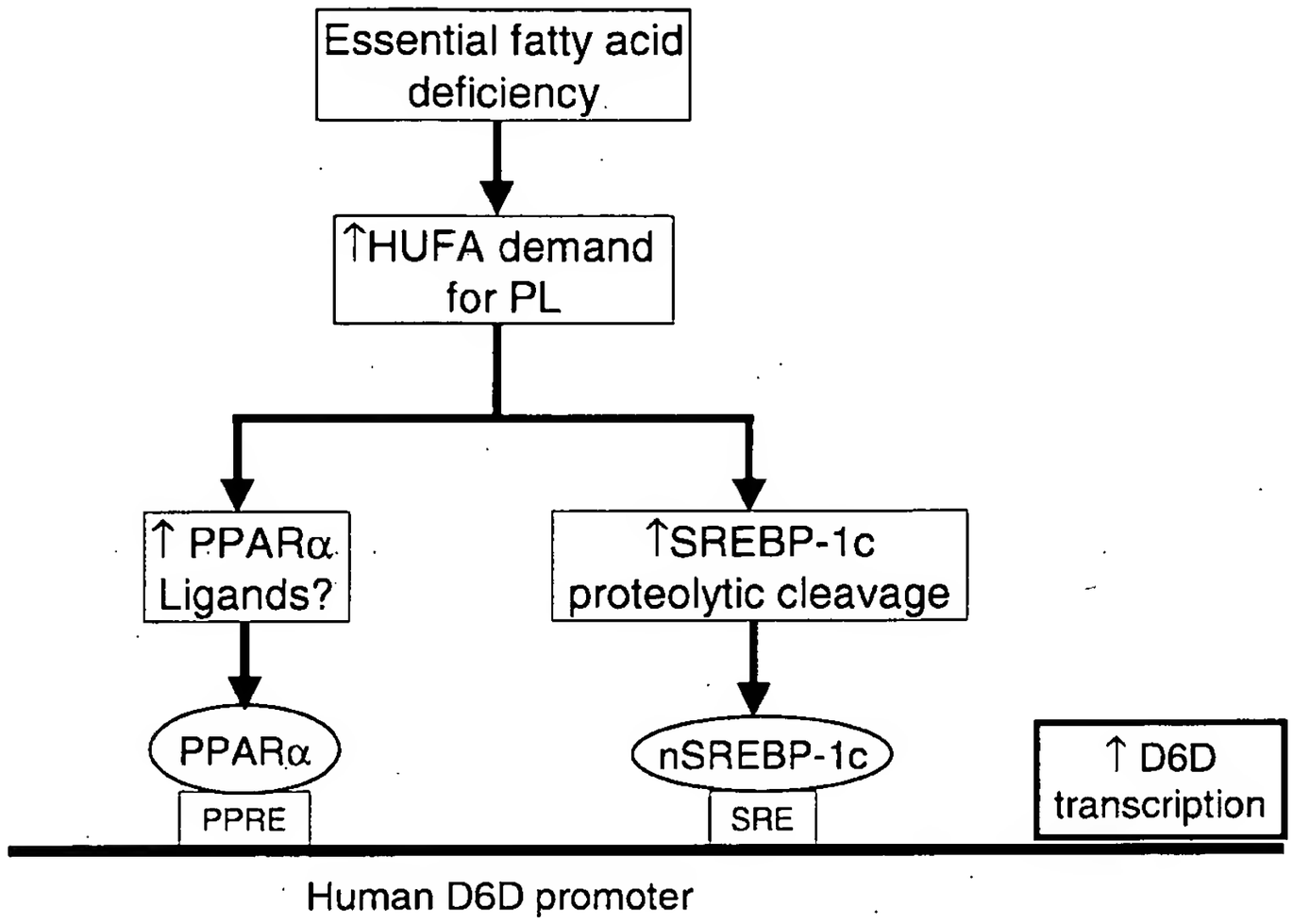

Figure 3 Proposed mechanism of *D6D* gene regulation.

induction of *D6D* when HUFAs are low. Moreover, PPARα-responsive genes such as *acyl CoA oxidase* and *CYP4A* are induced in wild-type mice fed EFA-deficient diets, which suggests endogenous ligands were generated under EFA deficiency (79). As summarized in Figure 3, available data suggest that two mutually antagonistic transcription factors (SREBP-1c and PPARα) act as sensors of HUFA status and together mediate feedback regulation of HUFA synthesis.

Regulation of Desaturases by Other Dietary Components and Hormones

ROLE OF SREBP-1c IN INDUCTION OF DESATURASES BY INSULIN Refeeding a high-carbohydrate diet after fasting rapidly increases SCD protein more than 40-fold in rodent liver, and the induction of *SCD* mRNA parallels its activity (120, 175). Also, both protein and mRNA of SREBP-1c decrease in fasting and increase rapidly in liver upon refeeding (46, 192). Disruption of the *SREBP-1c* gene blunted the induction of lipogenic genes including *SCD* mRNA in mouse liver upon refeeding (80), demonstrating that SREBP-1c at least in part mediates the induction of lipogenic genes. Injection of streptozotocin to rats dramatically decreased *SREBP-1c* mRNA, which was restored by insulin injection (161). Together, these studies suggest that SREBP-1c may mediate insulin effect on lipogenic genes. Consistent with this hypothesis, SREBP-1c binds the insulin response element in the *fatty acid synthase* gene (68, 84, 108). Studies with primary culture of rat hepatocytes showed that

expression of dominant negative SREBP-1c blocked the effect of insulin on transcriptional activation of genes involved in fatty acid synthesis, whereas expression of dominant positive SREBP-1c mimicked the insulin effect (26). The mechanism by which insulin induces *SREBP-1c* is yet to be elucidated.

Conserved SRE sequence GAT/ACAGCAGAG/T is present in the promoters of both *SCD* (3, 171, 198) and *D6D* (115) genes. Also, induction of *SCD* (185) and *D6D* mRNA (146) was diminished in diabetic rats and was restored by insulin administration. However, unlike *SCD*, the *D6D* mRNA was not affected by fasting/refeeding (39, 91). It is currently unknown whether this unresponsiveness is due to the longer half-life of *D6D* mRNA or to other mechanisms.

INDUCTION OF LIPOGENIC GENES BY CARBOHYDRATES AND THE ROLE OF ChREBP

Storing excess dietary carbohydrates as TGs is another important function of de novo lipogenesis. High glucose adds to the effects of insulin on the induction of glycolytic and lipogenic genes in liver. The carbohydrate response element (ChoRE) that mediates this glucose effect had long been identified in *liver-type pyruvate kinase* and *S-14* genes, although identity of the transcription factor that binds the element was elusive (61, 159). ChoRE consists of tandem E-box (CACGTG)-like sequences separated by five nucleotides. Although the E-box-like sequence suggested that a transcription factor of the bHLHLZ family bound ChoRE, known factors such as upstream stimulating factor did not bind the sequence (61). Recently, the transcription factor that mediates the glucose effect by binding ChoRE has been purified with affinity chromatography and named as carbohydrate response element-binding protein (ChREBP) (195). As predicted, ChREBP is a transcription factor of the bHLHLZ family (195). Under a high glucose condition, ChREBP is dephosphorylated and translocated to nucleus, resulting in the activation of target genes (58). Protein phosphatase 2A, which is activated by xylulose-5-phosphate (an intermediate of the pentose phosphate cycle), is capable of dephosphorylating ChREBP (56).

ChREBP is specifically expressed in liver (195) and adipose (76, 78), two major sites of TG synthesis, whereas SREBP-1c is widely expressed, including in tissues that lack a significant capacity of TG synthesis (162). Moreover, ChREBP was markedly induced when 3T3 preadipocytes differentiated into adipocytes (78), whereas SREBP-1c was not expressed in either 3T3 preadipocytes or adipocytes (162). These studies suggest that ChREBP, not SREBP-1c, plays the major role in the induction of lipogenic genes for storage of excess energy.

In addition to the *liver-type pyruvate kinase* gene, ChoRE has been identified in promoters of *fatty acid synthase* (148), *acetyl CoA carboxylase* (125), and *S14* (159). The presence of ChoRE in *fatty acid synthase* and *acetyl CoA carboxylase* is consistent with the report that these genes are still partially induced in the SREBP-1c-null mice upon refeeding (80). It is likely that ChREBP also regulates *SCD* because (*a*) oleic acid is the major constituent of TGs, and (*b*) disruption of SREBP-1c only partially reduced the induction of *SCD* mRNA upon refeeding (80). Fructose is more potent than glucose in inducing lipogenic mRNA such as

SCD (185) and *fatty acid synthase* (24). It has yet to be elucidated whether ChREBP mediates this fructose effect.

CHOLESTEROL SCD is also required for CE synthesis in liver (101). Dietary cholesterol induces SCD, whereas it depresses D6D and D5D (72, 73). LXR is the likely mediator of cholesterol effect on *SCD*. LXRs are transcription factors of the nuclear receptor family. Two isoforms have been identified: LXRα is the most abundant in liver; LXRβ is expressed ubiquitously (143). LXRs form heterodimers with retinoid X receptor and bind a direct repeat-4 element (LXR response element, LXRE) in target genes (21). Cholesterol metabolites, oxysterols, are its natural ligands and activate LXRs. LXRs activate a group of genes involved in reverse cholesterol transport (21, 143). In liver, activated LXRα induces the messages of *CYP7A* and *ATP-binding cassette transporters G5* and *G8* (143). Because CYP7A is an enzyme that catalyzes the rate-limiting step of bile acid synthesis, and ATP-binding cassette transporters G5 and G8 are cholesterol transporters, an overall effect of LXRα activation in liver is an increase in the bile acid synthesis and the secretion of cholesterol and bile acid to bile. When the LXRα gene is disrupted, the mouse becomes intolerant to dietary cholesterol and accumulates cholesterol in liver, underscoring the physiological function of LXRα (128). In addition, synthetic LXR agonists induce *SREBP-1c*, and LXRE is identified in the mouse *SREBP-1c* promoter (142). Thus, LXR agonists induce the lipogenic genes targeted by SREBP-1c, and increase production of very low-density lipoprotein in liver (32). A physiological role of this SREBP-1c induction by LXRα in liver may be providing fatty acids for the synthesis of PLs, which are also an essential component of bile.

Recently, LXRE was identified in the rat *fatty acid synthase* promoter (52). Also, dietary cholesterol was found to induce the *SCD-1* mRNA in an SREBP-1c-independent manner (63). These observations are consistent with a study of SREBP-1c-null mice in which an LXR agonist failed to induce *glucose-6-phosphate dehydrogenase* and *malic enzyme*, whereas residual induction was observed in *acetyl CoA carboxylase*, *fatty acid synthase*, and *SCD-1* by the LXR agonist (80). Therefore, like in the *fatty acid synthase* gene, the *acetyl CoA carboxylase* and *SCD-1* promoters may also have LXRE, and LXR may induce these genes both directly and indirectly via LXRE and SRE, respectively. Because CEs act as a reservoir when cholesterol is in excess, provision of fatty acids for CE synthesis may be the role for the SREBP-1c-independent induction of these three lipogenic genes by LXR. This explanation fits well with the finding that D6D and D5D were not induced by dietary cholesterol (72).

CONCLUSIONS

Recent cloning of desaturases in many species revealed the conservation of this group of enzymes across kingdoms, and indicated the essentiality of desaturases for free-living organisms. A common function of desaturases for organisms is

maintaining the physical property of membrane PLs and stored TGs. In addition, many animals and higher plants use PUFAs and metabolites for other functions, cell signaling in particular. Although eicosanoid functions are well characterized, mechanisms of many other functions of PUFAs are yet to be elucidated. Three mammalian desaturases share an overlapping function of maintaining unsaturated fatty acids in PLs. SCD is also required for TG and CE synthesis in liver, whereas D6D and D5D have a role in HUFA synthesis for a variety of cellular functions. Reflecting their functions, the regulation of three desaturases demonstrates shared as well as distinct mechanisms. Recent progress in elucidating regulatory mechanisms of desaturases presents exciting examples of the sophisticated control of mammalian gene transcription that is achieved by a combination of multiple transcription factors such as SREBP-1c, PPARα, ChREBP, and LXR.

ABBREVIATIONS

16:0, palmitic acid; 16:1 n-7, palmitoleic acid; 18:0, stearic acid; 18:1 n-9, oleic acid; 18:2 n-6, linoleic acid; 18:3 n-3, α-linolenic acid; 20:4 n-6, arachidonic acid; 22:6 n-3, docosahexaenoic acid; ACP, acyl carrier protein; bHLHLZ, basic helix-loop-helix leucine zipper; CE, cholesteryl ester; ChREBP, carbohydrate response element-binding protein; ChoRE, carbohydrate response element; CYP, cytochrome P450; D5D, Δ5 desaturase; D6D, Δ6 desaturase; D9D, Δ9 desaturase; EFA, essential fatty acid; ER, endoplasmic reticulum; HUFA, highly unsaturated fatty acid; LXR, liver X receptor; LXRE, LXR response element; MUFA, monounsaturated fatty acid; PL, phospholipid; PPARα, peroxisome proliferator activated receptor-α; PPRE, peroxisome proliferator response element; PUFA, polyunsaturated fatty acid; SCD, stearoyl CoA desaturase; SRE, sterol regulatory element; SREBP, SRE binding protein; TG, triglyceride.

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Hot Topic: Endogenous Production of n-3 and n-6 Fatty Acids in Mammalian Cells

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ABSTRACT

Polyunsaturated fatty acids (PUFA) are important components of mammalian diets, and the beneficial effects of n-3 PUFA on human development and cardiovascular health have been well documented. *Caenorhabditis elegans* is one of the few animals known to be able to produce linoleic (LA, 18:2n-6) and α-linolenic (ALA, 18:3n-3) essential fatty acids. These essential PUFA are generated by the action of desaturases that successively direct the conversion of monounsaturated fatty acids (MUFA) to PUFA. The cDNA coding sequences of the *C. elegans* Δ^{12} and n-3 fatty acid desaturases were each placed under the control of separate constitutive eukaryotic promoters and simultaneously introduced into HC11 mouse mammary epithelial cells by adenoviral transduction. Phospholipids from transduced cells showed a significant decrease in the ratios of both MUFA:PUFA and n-6:n-3 fatty acids relative to control cultures. The fatty acid profile of transduced cellular phospholipids revealed significant decreases in MUFA and arachidonic acid (20:4n-6), and increases in LA, ALA, and eicosapentaenoic acid (20:5n-3). The fatty acid composition of triacylglycerols derived from transduced cells was similarly, but less dramatically, affected. These results demonstrate the functionality of *C. elegans* fatty acid desaturase enzymes in mammalian cells. Expression of these desaturases in livestock might act to counterbalance the saturating effect that rumen microbial biohydrogenation has on the fatty acid profile of ruminant products, and allow for the development of novel, land-based dietary sources of n-3 PUFA.
(**Key words:** polyunsaturated fatty acid, n-3, fatty acid desaturase, transgenic)

Abbreviation key: ALA = α-linolenic acid, **CoA** = coenzyme A, **FAT-1** = n-3 fatty acid desaturase, **FAT-2** = Δ^{12} fatty acid desaturase, **LA** = linoleic acid, **MUFA** = monounsaturated fatty acids, **PUFA** = polyunsaturated fatty acids, **SFA** = saturated fatty acids.

Long-chain polyunsaturated fatty acids (**PUFA**) play a particularly important role in fetal development and the maintenance of overall human health, either as a component of membrane phospholipids, or as precursors to various eicosanoids (Jump, 2002). The most abundant PUFA are often described as n-3 (omega-3) or n-6 (omega-6), which refers to the number of the first carbon with a double bond relative to the carbon at the methyl end of the molecule. Because n-3 and n-6 PUFA are not interconvertible in mammals, elevating tissue concentrations of n-3 PUFA relies on chronic dietary intake of fats rich in n-3 PUFA. It has been observed that the ratio of n-6:n-3 PUFA present in the diet of industrial societies has increased as a result of the greater consumption of vegetable oils rich in n-6 fatty acids and a reduced consumption of fish and plant sources of n-3 fatty acids. This imbalance has been linked to an increased risk of coronary heart disease and other human ailments (Connor, 2000; Simopoulos, 2004). According to current dietary customs, the consumption of fish is the most practical source of long chain n-3 PUFA and concern exists as to whether the current fish-based supply is adequate to meet future needs (Pauly et al., 2002). To supply the requirements of a growing population, long-chain n-3 PUFA sources that are renewable and sustainable need to be developed.

Animal products represent a large proportion of the Western diet and are an important source of protein. Cattle contribute 36.1% of all protein consumed, whereas fish supply only 5.5% of total protein (Smit et al., 1999). Beef and dairy products contain low levels of PUFA because of the extensive fatty acid biohydrogenation carried out by the rumen microbial population. Increasing the proportion of PUFA in ruminant products could have significant human health benefits (Visioli et al., 2000). One approach to achieve this goal has been to treat dietary feedstuffs with heat or chemicals to protect the PUFA from rumen biohydrogenation (Noakes et al., 1996). This approach has met with only limited success and remains dependent upon the provi-

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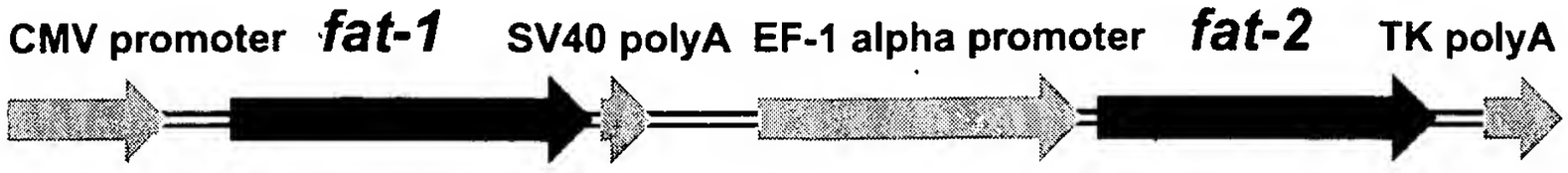

Figure 1. Schematic representation of the 5475-bp expression construct containing the *Caenorhabditis elegans* n-3 (*fat-1*; GenBank accession number L41807) and Δ^{12} (*fat-2*; GenBank accession number NM_070159) fatty acid desaturases under the control of constitutive eukaryotic promoters.

sion of exogenous PUFA in the feedstuff. An alternative approach to develop novel land-based sources of PUFA would be to genetically engineer animals to produce their own desaturase enzymes to allow for de novo fatty acid desaturation.

Vertebrates possess the stearoyl coenzyme A (**CoA**)-desaturase enzyme required to synthesize monounsaturated fatty acids (**MUFA**) from saturated fatty acids (**SFA**). However, they lack the fatty acid desaturase enzymes required for the synthesis of linoleic acid (**LA**, 18:2n-6) and α-linolenic acid (**ALA**, 18:3n-3), and are therefore dependent on dietary sources of these essential PUFA. The nematode *Caenorhabditis elegans* is able to synthesize both LA and ALA by virtue of an endogenous n-3 fatty acid desaturase (**FAT-1**) that recognizes a range of 18- and 20-carbon n-6 substrates (Spychalla et al., 1997) and a Δ^{12} fatty acid desaturase (**FAT-2**) that converts 16- and 18-carbon MUFA to n-6 fatty acids (Peyou-Ndi et al., 2000). We hypothesized that simultaneous expression of FAT-1 and FAT-2 in mammalian cells would enable the conversion of MUFA to n-3 PUFA and thus, the endogenous production of both LA and ALA essential fatty acids.

To test this hypothesis, FAT-1 and FAT-2 were constitutively expressed in HC11 mouse mammary epithelial cells. HC11 is a line of cells originally derived from COMMA-1D, an immortal cell line established from the mammary tissue of midpregnant BALB/c mice. This cell line can be grown using simplified cell culture conditions because it has no requirements for the addition of an exogenous extracellular matrix or co-cultivation with other cell types (Ball et al., 1988). Cells were maintained in growth medium consisting of RPMI+L-Glutamine (Gibco, Carlsbad, CA), supplemented with 8% heat-inactivated fetal calf serum (Gibco), 10 ng/mL epidermal growth factor (Sigma Chemical Co., St. Louis, MO), 5 μg/mL bovine insulin (Sigma Chemical Co.), and 50 μg/mL gentamicin (Gibco) in a humidified incubator at 37°C + 5% CO_2 (Marte et al., 1994). Cells were subcultured at a ratio of 1:5 every 3 d. HC11 cells were plated at 2×10^6 cells per 60-mm Petri dish on the day before infection with the prepared adenovirus.

To construct the adenoviral expression vector, the *C. elegans fat-1* (GenBank accession number L41807) and *fat-2* (GenBank accession number NM_070159) cDNA sequences were placed under the control of the CMV and EF-1 alpha constitutive promoters in pBudCE4.1 (Invitrogen, Carlsbad, CA) respectively (Figure 1), and subcloned into the Gateway pENTR 2B Vector (Invitrogen). A recombination reaction between the *att*L and *att*R attachment sites was performed to insert the desaturase genes into the pAd/CMV/V5/DEST Gateway Vector (Invitrogen) containing the human adenoviral gene, resulting in the pAd/*fat-1*/*fat-2* construct.

The 293A cell line of human embryonic kidney cells expressing the human adenovirus E1 proteins (Invitrogen) was used to produce the recombinant adenovirus. The cells were maintained in complete growth media consisting of DMEM (high glucose) (Gibco), 10% heat inactivated fetal bovine serum (Gibco), 0.1 m*M* MEM Nonessential Amino Acids (Gibco), 2 m*M* L-glutamine (Gibco), and 1% penicillin-streptomycin (Gibco) in a humidified incubator at 37°C + 5% CO_2. Cells were subcultured at ~75 to 85% confluence and fed complete growth media every 3 d. Cell density and viability were determined with a hemocytometer chamber and trypan blue exclusion (Graham et al., 1977).

To produce the adenovirus, both pAd/*fat-1*/*fat-2* and a control plasmid (pAd/CMV/V5-GW/*lac*Z, Invitrogen) were digested with *Pac*I to expose left and right viral inverted terminal repeats. Fragments were purified with a QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). The 293A cells were plated in 6-well plates at 5×10^5 cells/well. The following day, at approximately 90% confluency, the cultures were transfected with pAd/*fat-1*/*fat-2* or pAd/CMV/V5-GW/*lac*Z using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol with 3 μL of Lipofectamine 2000 and 1 μg of DNA per well. At 48 h post-transduction, the cells were transferred to a T-75 flask with complete growth media. Media were replaced every 3 d until cytopathic effects were observed, which was at approximately 12 d post-transduction. Approximately 18 d post-transduction, cytopathic effects had spread to 80% of the culture and plaques were visible. The cells and culture media were removed from the flasks and subjected to 3 rounds of freeze/thaw at –80°C (30 min) and 37°C (15 min). The cell lysate was centrifuged, and the supernatant containing the Ad/*fat-1*/*fat-1* or Ad/lacZ viral lysate was aliquoted, stored at –80°C, and titered before use.

HC11 cells were infected with either the Ad/*fat-1/fat-2* or the Ad/*lacZ* virus at a multiplicity of infection of 10. One culture transduced with Ad/*lacZ* virus was stained 3 d post-transduction using a β-galactosidase staining kit (Mirus, Madison, WI) to determine the efficiency of transduction (Figure 2). The adenoviral system resulted in a very high efficiency (~95%) of transduction and was found to be much more effective as a means of gene delivery to HC11 cells than lipofection (data not shown).

Three days post-transduction, the infected HC11 cells were rinsed with PBS (Gibco) and homogenized. Expression of FAT-1 protein in cultures transduced with Ad/*fat-1/fat-2* virus was confirmed by Western analysis (data not shown). Lipids were extracted in chloroform-methanol (2:1, vol/vol) containing 0.2% glacial acetic acid and dried under nitrogen at 60°C; the lipids were resuspended in hexane. Separation of polar and nonpolar lipids was performed with a Sep-pak silica cartridge WAT43400 (Waters, Milford, MA). Nonpolar lipids were eluted with hexane:ethyl ether (1:1, vol/vol). Polar lipids were eluted with methanol and chloroform:methanol:water (3:5:2, vol:vol:vol). Both samples were evaporated under nitrogen at 60°C and resuspended in 2 mL of iso-octane. The fatty acid profiles of the lipid fractions were analyzed via gas chromatography (DePeters et al., 2001).

Statistical analysis of cellular fatty acid profiles from 6 experimental replicates per virus treatment (Ad/*fat-1/fat-2* or the Ad/*lacZ* virus) was performed using a 1-way ANOVA. To examine the effect of the desaturase transgenes on the ratios of MUFA (16:1 + 18:1):PUFA (18:2 + 18:3 + 20:4 + 20:5) and n-6 (18:2 + 20:4):n-3 (18:3 + 20:5) fatty acids, the variance and covariance of the ratios were used to calculate the standard error of the difference between ratios (Cochran, 1977). A one-tail test of the hypothesis of the difference between the 2 ratios was performed using the calculated standard error: $R - R' \pm 1.65 \times SE(R - R')$ where R and R′ represent the ratios being compared, $SE(R - R')$ the standard error of the difference between the 2 ratios, and $1.65 = z_{0.05}$.

Phospholipids from mammalian cells expressing FAT-1 and FAT-2 showed a significant ($P < 0.05$) decrease in the ratios of both MUFA:PUFA (1.08 vs.1.81) and n-6:n-3 fatty acids (0.81 vs. 6.81) relative to the ratios found in control cultures expressing lacZ, respectively. The fatty acid profile of cells expressing FAT-1 and FAT-2 revealed an increase in LA, reflecting the known Δ^{12}-desaturase activity of FAT-2; and both a decrease in arachidonic acid (20:4n-6) and increases in ALA and eicosapentaenoic acid (20:5n-3), reflecting the known n-3 desaturase activities of FAT-1 (Figure 3a). Although LA is a substrate for FAT-1, the decrease in LA due to desaturation by FAT-1 was likely overshadowed by the influx of LA from the desaturation of oleic acid by FAT-2. The fatty acid composition of triacylglycerols derived from transfected cells was similarly, but less dramatically, affected (Figure 3b).

Because mammals lack the desaturase enzymes necessary to synthesize LA and ALA, they are dependent upon dietary sources of these essential fatty acids. In this experiment, LA and ALA constituted a relatively small proportion (2.45%) of phospholipid fatty acids in cultures that were transduced with Ad/*lacZ* virus. However, in the cultures that were transduced with Ad/*fat-1/fat-2* virus, the combined proportion of LA and ALA in the phospholipid fatty acid pool was significantly higher (6.45%). These results suggest that the cultures that were expressing FAT-1 and FAT-2 were successfully synthesizing essential fatty acids endogenously, utilizing a fatty acid desaturation pathway that is normally absent from mammalian cells.

Unlike mammalian desaturases that act on fatty acyl-CoA triacylglycerol precursors (Pereira et al., 2003), FAT-1 and FAT-2 are hypothesized to be acyl-lipid desaturases. This may explain why the desaturases appeared to have a more immediate effect on the composition of phospholipid-bound acyl groups. This speculation is supported by the finding that the FAT-1 enzyme was found to desaturate the 18:2 of *Arabidopsis* membrane lipids (Spychalla et al., 1997), and by the fact that the LA product of a fungal Δ^{12} acyl-lipid fatty acid desaturase expressed in mammalian cells was exclusively located in the cellular phospholipid fraction (Kelder et al., 2001).

The principal route of triacylglyceride biosynthesis involves the transfer of acyl-CoA to a glycerol backbone via the Kennedy pathway (Kennedy, 1961), although triacylglycerols can also be synthesized from the products of phospholipid hydrolysis. Transgenic mice constitutively expressing *fat-1* were found to have a significant decrease in the n-6:n-3 fatty acid ratio of milk fat (Kang et al., 2004), suggesting the movement of n-3 fatty acids from phospholipids into the triacylglycerol pool (~98% of milk fat). Interestingly, there were no apparent health problems in these transgenic mice although some may have been anticipated given the importance of phospholipid membrane composition on cellular metabolism (Hulbert, 2003). Imbalances in PUFA are attributed to many chronic disease states (Sanders, 1993), and can influence prostaglandin synthesis and fertility (Abayasekara and Wathes, 1999; Robinson et al., 2002). In vivo experiments will be required to establish what impact the coexpression of FAT-1 and FAT-2 desaturase enzymes might have on animal health and the PUFA content of milk and meat products derived from ruminant livestock.

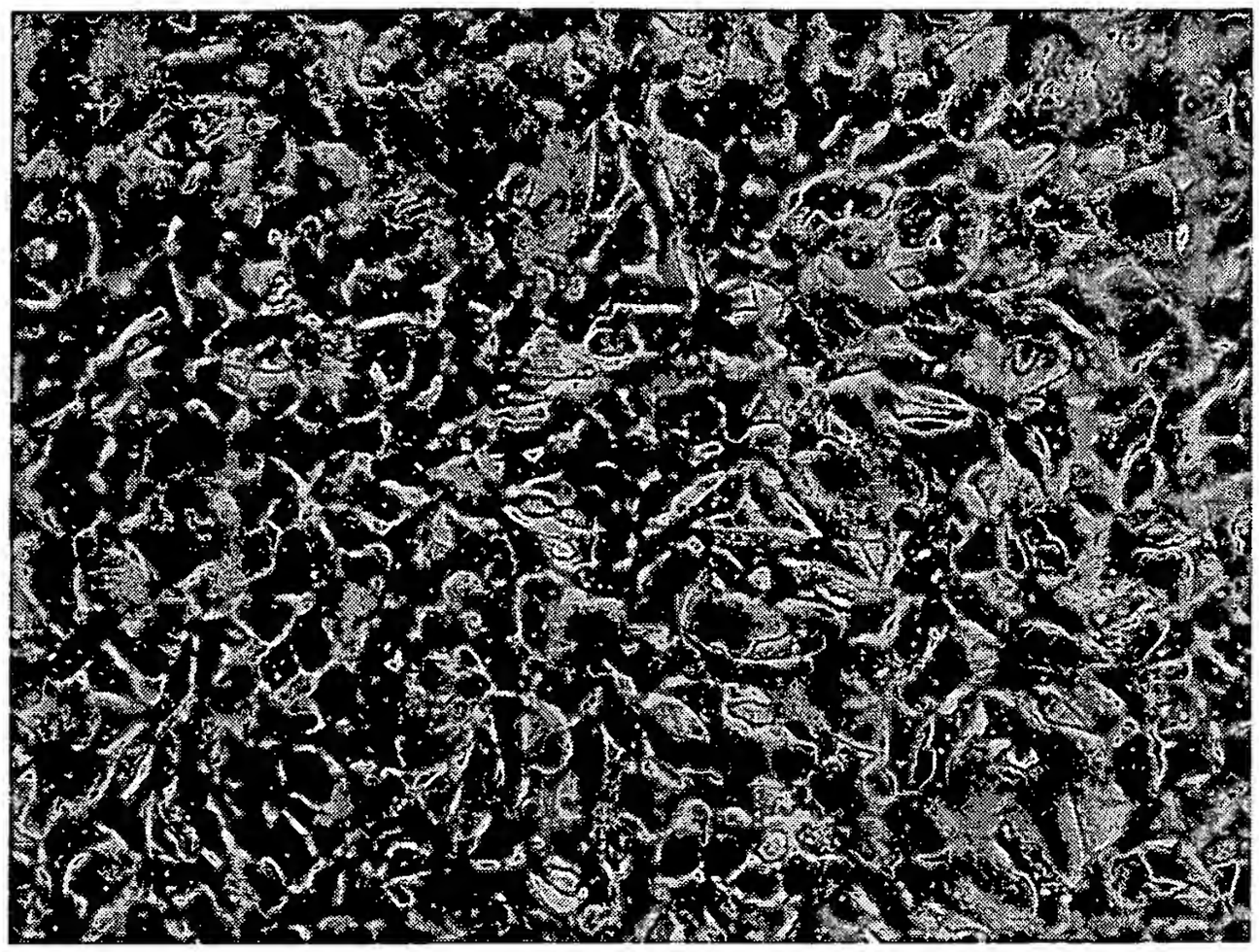

Figure 2. HC11 cells 3 d post-transduction with Ad/LacZ stained with β-galactosidase. Blue cells indicate successfully transfected cells showing a transduction efficiency of about 95% (20× magnification).

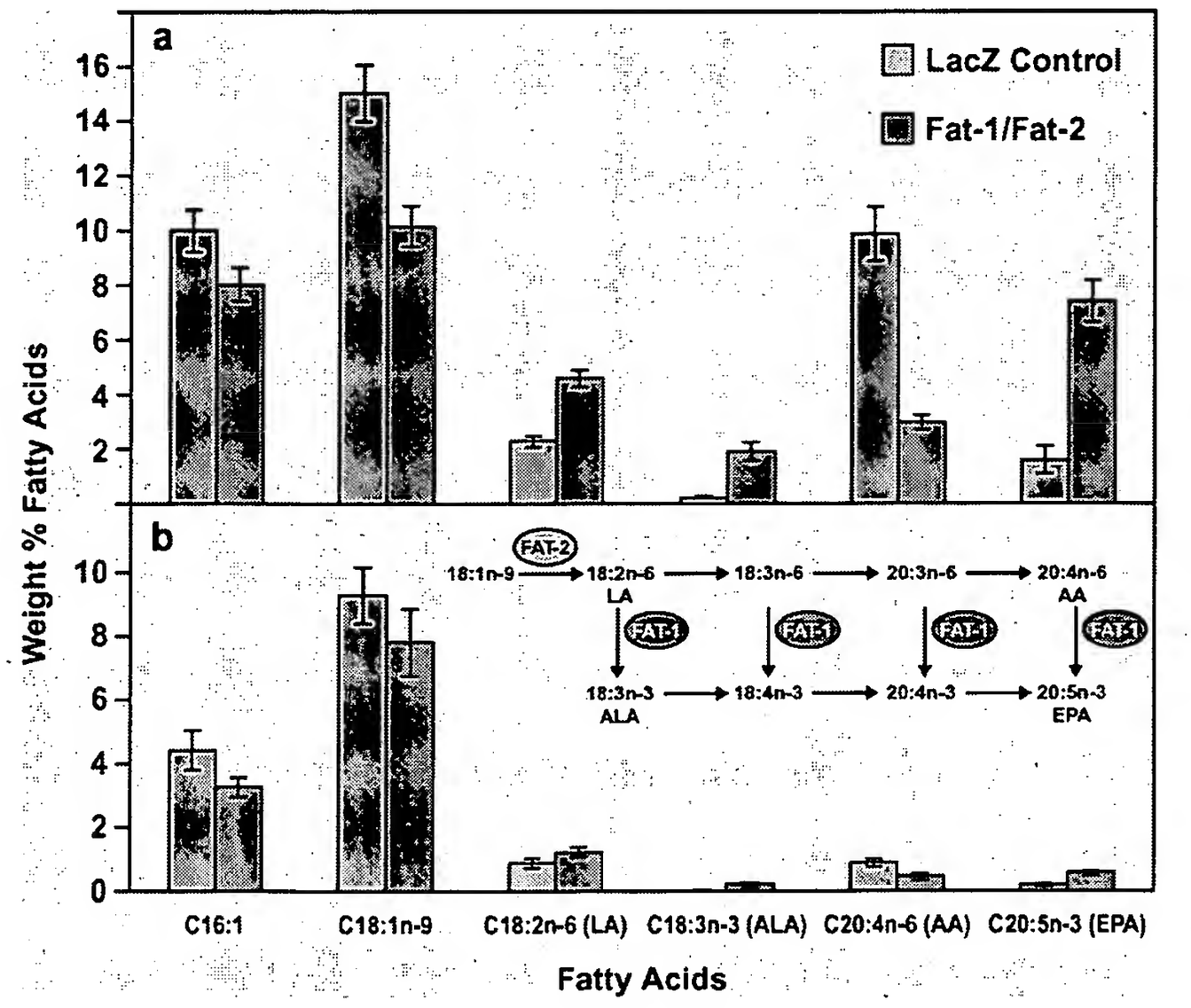

Figure 3. Fatty acid profile of cellular lipids as determined by gas chromatography. Fatty acid composition of the (a) phospholipid, and (b) triacylglycerol fraction of HC11 mouse mammary epithelial cells assayed 3 d after adenoviral-mediated transduction with recombinant virus containing *Caenorhabditis elegans* Δ^{12}-(FAT-2) and n-3 (FAT-1) fatty acid desaturases or LacZ under the control of constitutive promoters. Error bars represent SEM (n = 6). Inset: FAT-1 and FAT-2 mediated fatty acid desaturation pathways. LA = linoleic acid, ALA = alpha-linolenic acid, AA = arachidonic acid, EPA = eicosapentaenoic acid.

This study demonstrates that the expression of *C. elegans* fatty acid desaturase genes in mammalian cells can significantly alter the cellular fatty acid profile and suggests a transgenic approach to counteract the rumen microbial biohydrogenation of unsaturated fatty acids by enabling the endogenous production of PUFA. Increasing the n-3 PUFA of beef and dairy products offers a way to improve the nutritional content of an important component of the Western diet within the realm of existing food preferences, and would provide a compelling example of how biotechnology could be employed to produce functional foods for the enhancement of human health.

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Transgenicmice

Fat-1 miceconvert *n*-6to *n*-3fattyacids

Mammals cannot naturally produce omega-3 (*n*-3) fattyacids—beneficialnutrientsfoundmainlyinfishoil —fromthemoreabundantomega-6 (*n*-6) fattyacidsandsotheymustrelyonadietary supply[1]. Herewe showthatmiceengineered to carry a *fat-1* gene from the roundworm *Caenorhabditis elegans* can add a double bond into an unsaturated fatty-acid hydrocarbon chain and convert *n*-6 to *n*-3 fatty acids. This results in an abundance of *n*-3 and a reduction in *n*-6 fatty acids in the organs and tissues of these mice, in the absenceof dietary *n*-3. Aswellaspresenting anopportunitytoinvestigatetherolesplayed by *n*-3fattyacidsinthebody, ourdiscovery indicates that this technology might be adapted to enrich *n*-3 fatty acids in animal productssuchasmeat, milkandeggs.

The *fat-1* geneof *C. elegans* encodesan *n*-3 fatty-acid desaturase enzyme that converts *n*-6 to *n*-3 fatty acids and which isabsentinmostanimals, includingmammals[2,3]. We transferred this *fat-1* gene into mice and raised them alongside wild-type mice maintained on an identical diet that was high in *n*-6 but deficient in *n*-3 fatty acids. However, thefatty-acidprofilesof the twogroupsturnedouttobequitedifferent (Fig. 1). The tissues of wild-type animals containpolyunsaturatedfattyacidsthatare mainly(about98%) *n*-6linoleicacid(designatedan18:2 *n*-6fattyacidasithas18carbon atomsand2doublebonds, oneatposition *n*-6) and arachidonic acid (AA, 20:4 *n*-6), withverylittle *n*-3fattyacid(fromadietary source). Bycontrast, thetransgenicanimal tissuesarerichin *n*-3polyunsaturatedfatty acids, including linolenic acid (18:3 *n*-3), eicosa-pentaenoic acid (EPA, 20:5 *n*-3), docosa-pentaenoicacid(DPA, 22:5 *n*-3)and docosahexaenoicacid(DHA, 22:6 *n*-3).

The concentrations of *n*-6 linoleic and arachidonicacidsinthetissuesof thetransgenicmicearesignificantlyreduced, indicatingthat *n*-6fattyacidshavebeenconverted to *n*-3, causingtheratioof *n*-6to *n*-3todrop from20–50toalmost1. This *n*-3enrichment attheexpenseof *n*-6givesabalancedratioof *n*-6to *n*-3andof AA/(EPA+DPA+DHA)in allof theorgansandtissuestested(Table1). Transgenic skeletal muscle contains more EPAthanDHA, butDHAisthedominant *n*-3fattyacidinotherorgans.

We have examined the tissue fatty-acid profiles in four generations of transgenic mouselines (homozygoteorheterozygote) andfindconsistentlyraised *n*-3fattyacids, indicatingthatthetransgeneisfunctionally active *invivo* andtransmittable. Thetransgenicmiceappeartobenormalandhealthy.

Effortshavebeenmadetoincorporate *n*-3 fattyacidsintothefoodsupply[1,4] becauseof theirhealthbenefitsandconcernoverthehigh *n*-6:*n*-3ratioinWesterndiets. Ourfindings suggestanewstrategyforproducingfoodthat isenrichedin *n*-3fattyacidsfromlivestock carryingan *n*-3desaturasetrans-gene. Atpresent, farmanimalsarefedfishmealandother marineproducts, butthisistime-consuming andcostly, andislimitedbythequantityof the source[5]. Productionof *n*-3fattyacidsbythe animalsthemselveswouldbeacost-effective andsustainablewayof meetingtheincreasing demand; the ideal *n*-6:*n*-3 ratio of about 1 could be achieved by consuming foods containingthisratioandwithoutintroducing stringentdietarychanges.

Our transgenic mice also offer a model forinvestigatingthebiologicalfunctionsof *n*-3 fatty acids and the importance of the ratio of *n*-6:*n*-3 in disease prevention and treatment. Specificeffectsof *n*-3fattyacids andof the *n*-6:*n*-3ratiocanbetestedindifferent organs and tissues — for example, theymayaltergeneexpressionorphysiologicalactivityduringthelifecycle. Ourmouse linescouldbegeneticallybackcrossedwith mouse disease models to test the effects of *n*-3fattyacidsonthepathogenesisandtreatmentof thosediseases.

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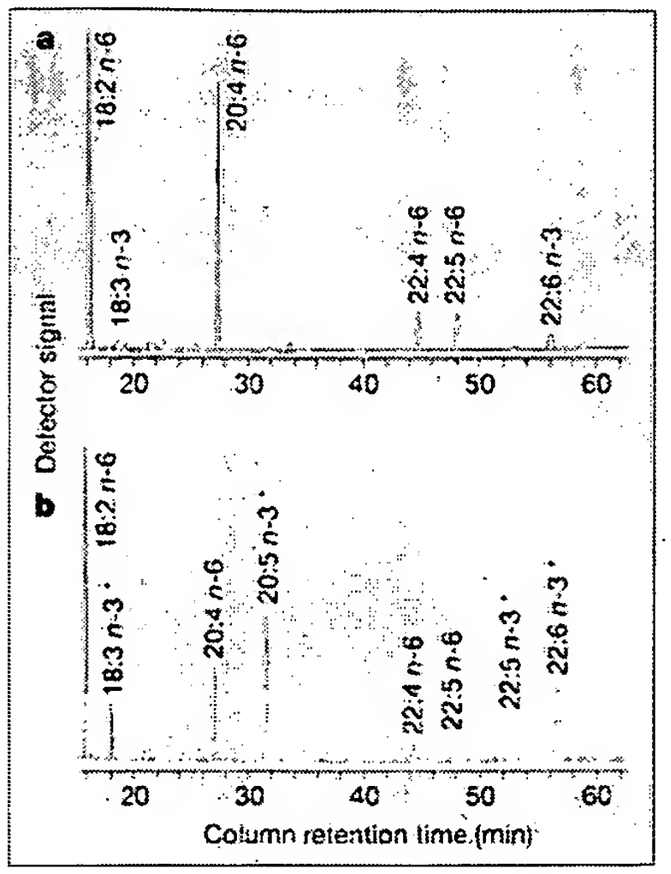

Figure1 Partialgaschromatographtracesshowingthepolyunsaturated fatty-acid profiles of total lipid extracted from mouse skeletalmuscle. **a, b**, Tracesfromlipidfrom **a**, awild-typemouse, and **b**, a *fat-1* transgenic mouse (heterozygote). Theexpression vector used for microinjection contained the humanized *fat-1* sequence (with modification of codon usage) and a chicken β-actin promoter and cytomegalovirus enhancer, which allow high and broad expression of the transgene in mice[6,7]. Both the wild-type and transgenic mice were 8-week-old females that were fed onthesamediet, whichwashighin *n*-6butlowin *n*-3fattyacids. Thelipidprofilesshowthatconcentrationsof *n*-6polyunsaturated acids(18:2 *n*-6, 20:4 *n*-6, 22:4 *n*-6and22:5 *n*-6)arelowerand levelsof *n*-3fattyacids(asterisks)aremarkedlyhigherintransgenic (**b**) than in wild-type (**a**) muscle. (Homozygotes and heterozygoteshaveasimilarphenotype.)

Table1 **Fatty-acidratiosinWTand *fat-1* mice**

	*n-6/n-3**		*AA/(EPA+DPA+DHA)*	
	WT	*TM*	*WT*	*TM*
Muscle	49.0	0.7	11.3	0.4
Milk†	32.7	5.7	15.7	2.5
Erythrocyte	46.6	2.9	27.0	1.6
Heart	22.8	1.8	14.3	0.9
Brain	3.9	0.8	3.6	0.7
Liver	26.0	2.5	12.5	0.9
Kidney	16.5	1.7	11.9	1.2
Lung	32.3	2.2	19.8	1.2
Spleen	23.8	2.4	17.3	1.5

Bothwild-type(WT)andtransgenic(TM)micewere8-week-oldfemalesfed onthedietdescribedinFig.1. AA, arachidonicacid(20:4 *n*-6);EPA, eicosapentaenoicacid(20:5 *n*-3);DPA, docosapentaenoicacid(22:5 *n*-3); andDHA, docosahexaenoicacid(22:6 *n*-3).

*The *n*-6:*n*-3fatty-acidratioisgivenby(18:2 *n*-6 + 20:4 *n*-6 + 22:4 *n*-6 + 22:5 *n*-6):(18:3 *n*-3 + 20:5 *n*-3 + 22:5 *n*-3 + 22:6 *n*-3).

†Themilkwastakenfromthestomachcontentsof5-day-oldneonatalmice borntowild-typeortransgenicmothers.

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the standard deviation does not. This is because changing the units corresponds to multiplying the data with a constant (a), which adds a constant to the log values: $\ln aN_i = \ln a + \ln N_i$. The resulting change in 'synchrony' depends both on $\text{stdev}_{\text{all } i}(\ln N_i)$, which is specific to each year's set of abundances, and on the choice of unit (specifically, $\text{sync}(aN) = \text{sync}(N) + \ln(a)/\text{stdev}_{\text{all } i}(\ln N_i)$). Therefore, a change of unit may qualitatively reverse comparisons of synchrony between data sets.

This problem is not resolved by addition of a constant to all log-transformed musk-ox abundances to ensure that these are positive before calculating 'synchrony' (Post and Forchhammer, personal communication), as the relationship between mean ln aN and stdev ln aN remains entirely arbitrary. Biological conclusions should not be affected by whether American, metric or other units are used.

Post and Forchhammer's analyses show an apparent tendency for cross-species correlation to decrease with increasing interpopulation distance. However, the few strong correlations describe concurrent trends over decades, rather than the year-to-year variation that was Post and Forchhammer's focus. Plotting cross-species correlation against each ($\omega^{(i)}_{\text{musk ox}}$, $\omega^{(j)}_{\text{caribou}}$) pair shows no consistent pattern. We also correlated growth rates instead of raw abundances and found unsystematic and weak correlations. Although other approaches might be more successful, the data may not be sufficiently precise or relevant to detect the phenomenon if it exists. We conclude that there is currently no proper evidence of climate-induced synchrony between musk oxen and caribou on Greenland.

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Post and Forchhammer reply — Vik *et al.* question whether we documented spatial synchrony between caribou and musk oxen from Greenland, and whether spatial synchrony within each species related to the North Atlantic Oscillation (NAO)[1]. Attributing spatial synchrony to climate is difficult but possible[2], and the questions raised by Vik *et al.* are readily addressed. Contrary to their incorrect statement of our definition of the NAO effect ratio[1], a strong climatic effect on any pair of populations is not a requisite of climate-induced synchrony. As Moran[3] argued, and as our analysis illustrated[1], populations may be synchronized if climate influences each of them similarly, regardless of the magnitude of that influence. Moreover, the standardized NAO effect ratio is associated statistically with the degree of climatic correlation across populations[4] and hence the degree of synchrony between populations[3].

As stated previously[1], we used cross-population covariance (CV) to produce a time-series index of spatial synchrony, an approach validated in empirical[5] and theoretical[6] studies, which have demonstrated the relationship of CV to population synchrony[7]. The simplest test of whether the use of log-transformed data confounds our results is to compare them with results obtained using raw (not log-transformed) data. The correlation between the NAO and 1/CV of the raw musk-ox data[8] ($r = -0.57, P = 0.001$) matches exactly the correlation between the NAO and 1/CV of the log-transformed musk-ox data ($r = -0.59$, $P = 0.001$). Similarly, results do not vary for caribou, using log-transformed ($r = 0.35$, $P = 0.002$) or raw ($r = 0.24$, $P = 0.04$) data. Hence, log-transformation does not influence relationships between the NAO and spatial synchrony.

Moreover, our results were not influenced by addition of a constant to the log-transformed musk-ox data, which Vik *et al.* describe as analogous to changing units of abundance. Such a problem would be apparent if the means of the N_i, $\ln(N)_i$, or $[\ln(a) + \ln(N)_i]$ showed significant and inconsistent correlations with the NAO, but none did (r_i values of 0.14, 0.07 and 0.07, respectively; all P values ≥ 0.50). Vik *et al.* obtained different results because their direct log-transformation of the decimal-form musk-ox data produced negative values, giving statistically invalid CVs[9,10]. We added 4 to the log-transformed musk-ox data to convert negative values to positives before calculating the CV precisely to avoid a spurious correlation.

If the NAO–spatial synchrony correlation were, in fact, influenced by the use of log-transformed data, such an artefact should be apparent in two ways, neither of which is discussed by Vik *et al.* First, the sign of the correlation between the NAO and musk-ox spatial synchrony should change with addition of constants greater than 4. Second, adding constants to log-transformed caribou data (to which none was originally added[1]) should also alter the NAO–caribou synchrony correlation. We checked this by adding constants of up to 10, and in neither case was the correlation altered (Fig. 1).

We conclude that our previous results still stand and that Vik *et al.* cannot offer a means of analysing or an alternative explanation for spatial synchrony within and across these species.

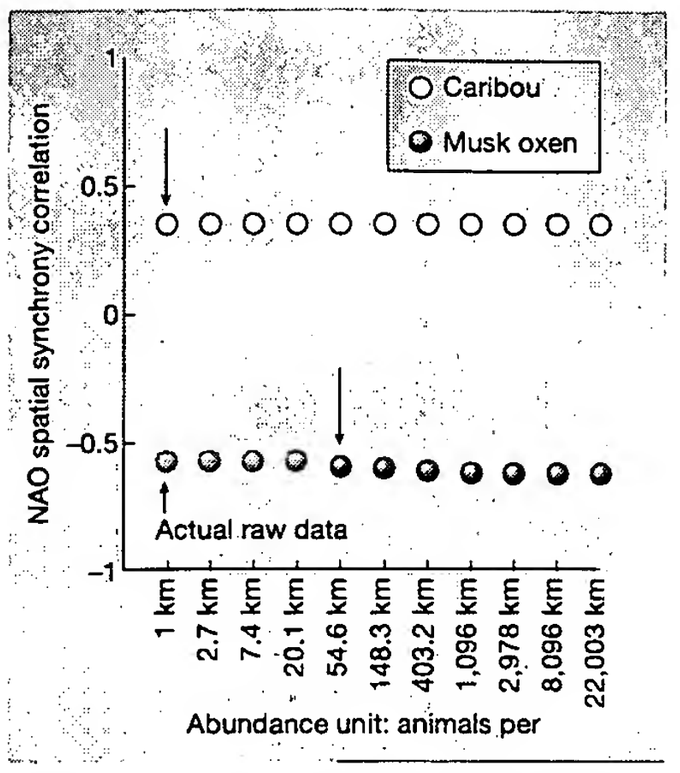

Figure 1 Addition of constants from zero to ten (corresponding to abundance units shown on the *x*-axis) to ln-transformed density estimates does not influence the sign of the correlation between the NAO index and spatial synchrony for musk oxen or caribou. Points designated by an arrow are the correlations reported in our original analysis[1]. For the ln-transformed musk-ox data, addition of constants less than four, as done by Vik *et al.*, produces spurious correlations because the resulting negative values do not give statistically valid coefficients of variation[9,10]. Thus, the blue points indicate the correlations obtained when using the raw musk-ox data[8] with constants of zero to three added; note that the point denoted as raw data in Fig. 1 of Vik *et al.* is in fact a direct ln-transformation of the actual raw data.

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corrigendum

***Fat-1* mice convert *n*-6 to *n*-3 fatty acids**

Jing X. Kang, Jingdong Wang, Lin Wu, Zhao B. Kang
Nature **427**, 504 (2004)

J. X. K. is the inventor and co-applicant (with Massachusetts General Hospital, a non-profit organization) of a patent application relevant to this work (USSN 60/275,222: WO02072028), which should therefore have been declared as a competing financial interest.

SECOND EDITION

Transgenic Animal Technology

A Laboratory Handbook

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Conjugated Linoleic Acid Is Synthesized Endogenously in Lactating Dairy Cows by Δ^9-Desaturase[1,2]

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ABSTRACT Conjugated linoleic acid (CLA) is a naturally occurring anticarcinogen found in milk fat and body fat of ruminants. Although CLA is an intermediate in ruminal biohydrogenation of linoleic acid, we hypothesized that its primary source was from endogenous synthesis. This would involve Δ^9-desaturase and synthesis from *trans*-11 18:1, another intermediate in ruminal biohydrogenation. Our first experiment supplied lactating cows (n = 3) with *trans*-11 18:1 by abomasal infusion and examined the potential for endogenous synthesis by measuring changes in milk fat CLA. By d 3, infusion of *trans*-11 18:1 resulted in a 31% increase in concentration of *cis*-9, *trans*-11 CLA in milk fat, demonstrating that an active pathway for endogenous synthesis of CLA exists. Our second experiment examined the quantitative importance of endogenous synthesis of CLA in lactating cows (n = 3) by abomasally infusing a putative stimulator (retinol palmitate) or an inhibitor (sterculic oil) of Δ^9-desaturase. Infusion of retinol palmitate had no influence on milk fatty acid desaturation, and yield of CLA in milk fat was not altered. However, sterculic oil infusion decreased the concentration of CLA in milk fat by 45%. Consistent with Δ^9-desaturase inhibition, the sterculic oil treatment also altered the milk fat concentration of other Δ^9-desaturase products as indicated by the two- to threefold increase in the ratios of 14:0 to 14:1, 16:0 to 16:1 and 18:0 to *cis*-18:1. Using changes in the ratio of 14:0 to 14:1 as an indication of the extent of Δ^9-desaturase inhibition with the sterculic oil treatment, an estimated 64% of the CLA in milk fat was of endogenous origin. Overall, results demonstrate that endogenous synthesis of CLA from *trans*-11 18:1 represented the primary source of CLA in milk fat of lactating cows. J. Nutr. 130: 2285–2291, 2000.

KEY WORDS: • *conjugated linoleic acid* • *Δ^9-desaturase* • *lactation* • *milk fat* • *ruminants*

Conjugated linoleic acid (CLA) has a wide range of physiologic effects in animal models [see reviews by Banni and Martin (1998) and Pariza (1999)]. Many of these may represent positive health benefits of dietary CLA. Dairy products are the major dietary source of CLA, and *cis*-9, *trans*-11 octadecadienoic acid is the predominant CLA isomer in natural lipids (Parodi 1997). A trivial name, rumenic acid, was proposed for this isomer on the basis of its ruminant origin (Kramer et al. 1998). The sequence of ruminal biohydrogenation of linoleic acid involves isomerization to form *cis*-9, *trans*-11 CLA followed by successive reductions to *trans*-11 octadecenoic acid (vaccenic acid) and stearic acid (Harfoot and Hazlewood 1988). On this basis, the CLA in milk fat and body fat of ruminants has been assumed to be CLA that has escaped complete biohydrogenation in the rumen (Chin et al. 1992, Parodi 1997 and 1999).

Concentrations of CLA in milk fat can be enhanced by changes in the diet, especially utilization of diets with greater linoleic acid content [see review by Griinari and Bauman (1999)]. However, certain diets that have low levels of linoleic acid, e.g., pasture or fish oil feeding, also increase the concentration of CLA in milk fat. These diets contain high levels of other polyunsaturated fatty acids (PUFA) that do not yield CLA as an intermediate in rumen biohydrogenation (Griinari and Bauman 1999, Harfoot and Hazelwood 1988). This raises the possibility of alternative sources of milk fat CLA. In the ruminal biohydrogenation of linoleic acid, CLA is a transient intermediate, whereas *trans*-11 18:1 accumulates (Harfoot and Hazlewood 1988). Furthermore, *trans*-11 18:1 is an intermediate in the biohydrogenation of several PUFA (Griinari and Bauman 1999). On this basis, we hypothesized that CLA could

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be produced by endogenous synthesis from *trans*-11 18:1 by Δ^9-desaturase (Griinari et al. 1997). Consistent with this, mammary gland and adipose tissue of ruminants have substantial Δ^9-desaturase activity (Kinsella 1972, Martin et al. 1999, St. John et al. 1991, Ward et al. 1998).

The objective of this investigation was to examine the endogenous synthesis of CLA in lactating dairy cows. The first experiment supplied *trans*-11 18:1 by abomasal infusion and examined the potential for endogenous synthesis by measuring changes in milk fat CLA. In the second experiment, we examined the quantitative importance of endogenous synthesis of CLA by inhibiting the activity of Δ^9-desaturase with sterculic oil. In this latter study, we also included a treatment with retinol palmitate, a compound that has been reported to enhance gene expression of Δ^9-desaturase in mouse liver.

MATERIALS AND METHODS

The Cornell University Institutional Animal Care and Use Committee approved all procedures involving animals. Experiments utilized multiparous Holstein cows fitted with rumen fistulas. Cows were maintained in metabolism stalls at the University's Large Animal Research and Teaching Unit. Total mixed diets were formulated using the Cornell Net Carbohydrate and Protein System (Fox et al. 1992) to meet or exceed predicted requirements (NRC 1989). Ingredients and chemical composition of the diet are presented in Table 1. Cows were fed for ad libitum intake with fresh feed being offered twice daily. The amount of feed consumed was measured daily and water was available at all times.

Cows were milked at 0600 and 1800 h each day. At each milking, yield was recorded and milk was sampled. One aliquot of milk was stored at 4°C with a preservative (Bronopol tablet; D&F Control System, San Ramon, CA) until analyzed by infrared analysis for fat and protein content (Northeast DHI, Ithaca, NY). A second aliquot without preservative was stored at −20°C until fatty acid analysis.

Treatments were infused into the abomasum. This is a convenient experimental method to simulate dietary supply of compounds while avoiding possible alterations by rumen bacteria. The abomasum was accessed by passing a polyvinyl chloride tube (0.5-cm i.d.) through the rumen fistula, rumen compartments and sulcus omasi, and into the abomasum as described previously (Spires et al. 1975).

TABLE 1

Ingredient and chemical composition of experimental diets

Composition	Experiment 1	Experiment 2
Ingredient	*g/100 g dry matter*	
Chopped alfalfa hay	47.0	45.1
Cracked corn	31.0	22.8
Soybean meal	12.6	7.0
Extruded soybeans	—	8.4
Soy plus	4.6	—
Citrus pulp	—	9.9
Whole cottonseed	—	4.4
Beet pulp	2.3	—
Mineral-vitamin supplement[1]	2.5	2.4
Chemical analysis[2]		
Crude protein	17.9	16.5
Crude fat	2.9	4.7
NDF[3]	29.5	41.1
	MJ/kg dry matter	
NE_L[4]	4.36	6.52

[1] Contained 385 g NaCl and 615 g of a trace mineral and vitamin mix per kg of supplement. Trace mineral and vitamin mix contained (g/kg mix) Mn, 1.1; Zn, 1.4; Fe, 0.50; Cu, 0.25; I, 0.027; Co, 0.024; Se, 0.007; retinyl acetate, 0.258; cholecalciferol, 0.007; and *dl*-α-tocopheryl acetate, 2.56.

[2] Analyses were by Northeast DHI (Ithaca, NY).

[3] Neutral detergent fiber.

[4] Net energy for lactation.

Experiment 1. The three cows averaged 152 ± 25 d postpartum (mean ± SD) at the start of the study. The 11-d experiment consisted of a pretreatment period (d 1–3), a treatment period (d 4–6), and a post-treatment period (d 7–11). Skim milk (vehicle) was infused abomasally during the pre- and post-treatment periods. During the treatment period, a *trans* fatty acid emulsion in skim milk was used. A mixture containing equal amounts of *trans*-11 and *trans*-12 octadecenoic acids (Lot #7363:10; Larodan Fine Chemicals, Malmö, Sweden) was used due to availability and cost. Company specifications indicated that the *trans*-11 and *trans*-12 18:1 were in equal ratio and comprised >99% of the fatty acids in the chemical mixture; this was confirmed by our own analysis. The *trans*-18:1 mixture was added to heated skim milk and an emulsion prepared using a microfluidizer as described by Chouinard et al. (1999). The final concentration of the *trans*-octadecenoic acid mixture was 0.5% in the skim milk emulsion.

Infusions used a peristaltic pump (Harvard Apparatus, South Natick, MA) calibrated to infuse continuously at a rate of 5 kg/d. This resulted in a delivery rate of 25.0 g/d of the *trans*-18:1 mixture during the treatment period. Sanitized carboys served as reservoirs for infusates, and were changed every 12 h.

Experiment 2. Three cows, 144 ± 94 d postpartum, were randomly assigned to a 3 × 3 Latin square design. Treatments were administered by abomasal infusion and included the following: 1) control (200 mL water/d), 2) retinol palmitate (4.8 g/d) and 3) sterculic oil (10 g/d). Equal volumes of the infusates were administered at 6-h intervals for 4 d with a 7-d interval between infusion periods.

For the retinol palmitate treatment, infusions were prepared as a suspension in water. Retinol palmitate (825,000 retinol equivalents/g) was obtained from Sigma-Aldrich (St. Louis, MO), and the final suspensions contained 24 g/L retinol palmitate. Cows were infused 4 times/d with 50 mL/infusion, resulting in a daily dosage of 4.8 g of retinol palmitate.

Sterculic oil was extracted from the seeds of the *Sterculia foetida* tree. Seeds were dehulled, crushed and the meats refluxed in diethyl ether to extract the oil (method 963.15; AOAC 1998). The yield of extracted oil was 49.6% of the seed meat by weight. The sterculic oil was prepared for abomasal infusion by making a 2% emulsion in skim milk as described for Experiment 1. Emulsions were stored at 4°C until infused, with fresh emulsions prepared for each treatment period. Cows receiving the sterculic oil treatment were infused with an equal amount 4 times/d. The daily dose averaged 9.7 g of sterculic oil and 468 mL of emulsion.

Fatty acid analysis. Lipid extraction of milk fat was performed according to Hara and Radin (1978). Methyl esters of the fatty acids were prepared by transesterification with sodium methoxide according to the method of Christie (1982) as detailed by Chouinard et al. (1999).

Fatty acid methyl esters were quantified by gas chromatography techniques. Two methods were used to allow complete separation of *trans*-11 and *trans*-12 octadecenoic acids and their respective desaturase products. One method used a CP-Sil 88 column (cyanopropyl polysiloxane; 100 m × 0.25 mm i.d. with 0.20-μm film thickness; Chrompack, Middlebury, The Netherlands) with two temperature-programmed gas chromatography runs. The first involved a temperature gradient program (70–240°C) and the second was an isothermal run at 160°C as described by Griinari et al. (1998). This method separated *trans*-11 and *trans*-12 octadecenoic acids into single-component peaks. The second method used a Supelcowax-10 column (fused silica, 60 m × 0.32 mm i.d. with 0.25-μm film thickness; Supelco, Bellefonte, PA) as described by Chouinard et al. (1999). This method provided data for *cis*-9, *trans*-12 octadecadienoic acid concentration, the general fatty acid composition of milk fat and the fatty acid composition of sterculic oil.

For both gas chromatography methods, fatty acids were identified using pure standards (Nu-Chek-Prep, Elysian, MN). A butter reference standard (CRM 164; Commission of the European Communities, Community Bureau of Reference, Brussels, Belgium) was used to

determine recoveries and correction factors for individual fatty acids in milk fat.

Statistical analysis. Data from Experiment 1 were analyzed using the general linear models procedure of SAS (1989) according to the following model:

$$Y_{ij} = \mu + T_i + C_j + E_{ij}$$

where Y_{ij} is the observation, μ is the overall mean, T_i is the treatment (i = 1 and 2), C_j is the cow (j = 1, 2 and 3) and E_{ij} is the residual error. Data from d 1 through 3 plus d 9 through 11 of the experimental period constituted the control values and data from d 6 (d 3 of treatment infusion) represented treatment values.

For Experiment 2, data were analyzed as a 3 × 3 Latin square design using the PROC MIXED procedure of SAS (1989) according to the following model:

$$Y_{ijk} = \mu + T_i + P_j + C_k + E_{ijk}$$

where Y_{ijk} is the observation, μ is the overall mean, T_i is the treatment (i = 1, 2 and 3), P_j is the period (j = 1, 2 and 3), C_k is the cow (k = 1, 2 and 3) and E_{ijk} is the residual error. Data from d 3 and 4 from each treatment period were used in the analysis.

RESULTS

Experiment 1. Our initial experiment infused *trans*-11 18:1 abomasally to examine the potential for endogenous synthesis of CLA by Δ^9-desaturase. Due to availability and cost we used a 50:50 mixture of *trans*-11 18:1 and *trans*-12 18:1. The Δ^9-desaturase could catalyze the formation of *cis*-9, *trans*-11 CLA and *cis*-9, *trans*-12 18:2 from *trans*-11 18:1 and *trans*-12 18:1, respectively. Cows maintained constant feed intake during the 11 d of the study (data not presented). The yields of milk and milk fat were also relatively constant throughout the study (**Fig. 1**). However, there were alterations in the pattern of milk fatty acids over the treatment period. Abomasal infusion of the mixture of *trans*-11 and *trans*-12 octadecenoic acids resulted in the appearance of these fatty acids in milk fat (**Fig. 2**). In addition, the respective *cis*-9, *trans*-n octadecadienoic acids formed from *trans*-11 and *trans*-12 octadecenoic acids by the action of Δ^9-desaturase

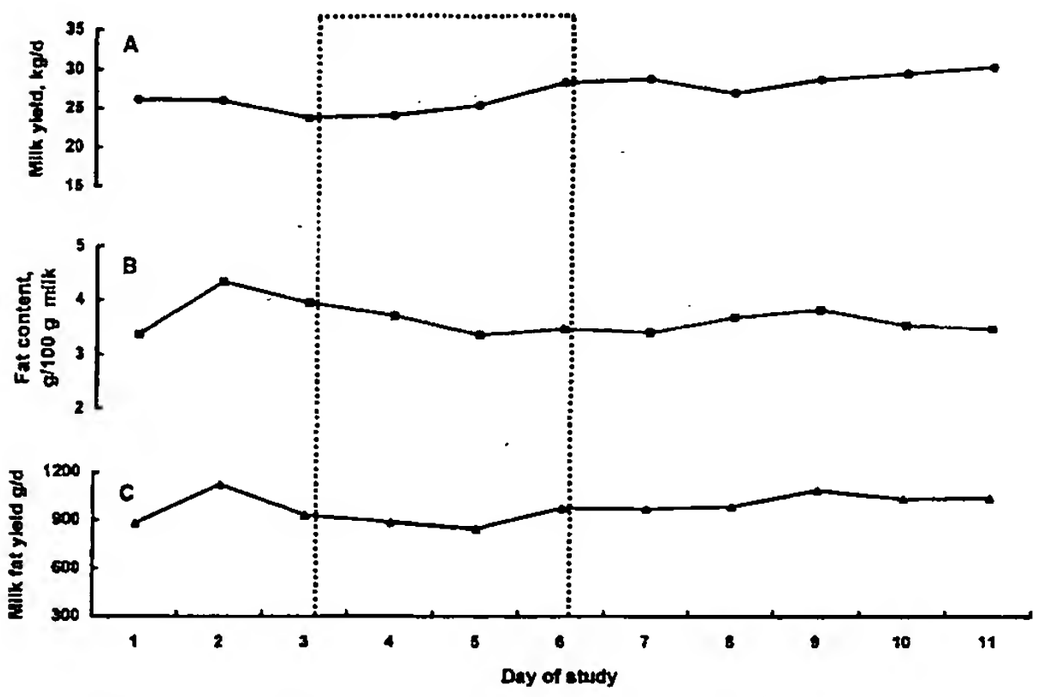

FIGURE 1 Temporal patterns for milk yield (*Panel A*), milk fat content (*Panel B*) and milk fat yield (*Panel C*) in lactating dairy cows receiving abomasal infusion of *trans*-11 and *trans*-12 18:1. The treatment period (indicated by dotted line) involved a 3-d abomasal infusion of *trans*-11 and *trans*-12 18:1 emulsified in skim milk. Vehicle was infused abomasally for the 3-d pretreatment and 5-d post-treatment periods. Values represent mean for 3 cows; the pooled SEM was 0.8 kg/d for milk yield, 0.08% for milk fat content and 26 g/d for milk fat yield.

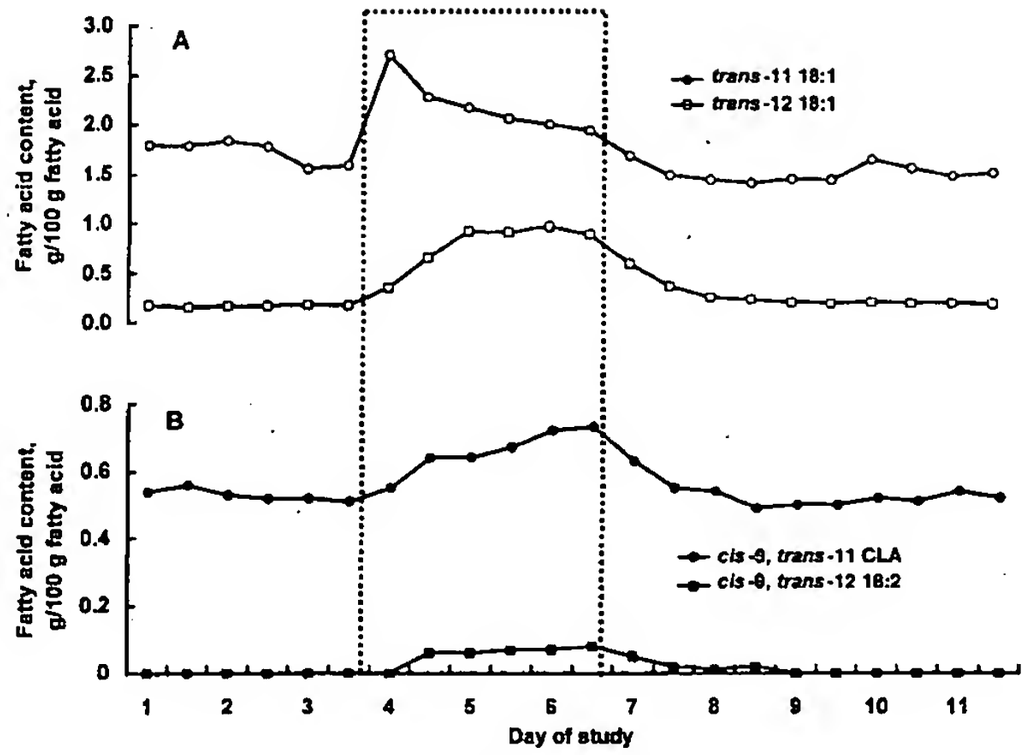

FIGURE 2 Temporal pattern of *trans*-11 and *trans*-12 18:1 (*Panel A*) and their Δ^9-desaturated dienes (*Panel B*) in lactating dairy cows receiving abomasal infusion of *trans*-11 and *trans*-12 18:1. The treatment period (indicated by dotted lines) involved a 3-d abomasal infusion of *trans*-11 and *trans*-12 18:1 (25 g/d) that commenced on d 4 and continued through d 6. Vehicle was infused abomasally during the pretreatment and post-treatment periods. Values represent mean for 3 cows; the pooled SEM was 0.12 g/100 g fatty acid for *trans*-11 18:1, 0.03 g/100 g fatty acid for *trans*-12 18:1, 0.02 g/100 g fatty acid for *cis*-9, *trans*-11 CLA and 0.01 g/100 g for *cis*-9, *trans*-12 18:2. The analytical methods involved the use of pure standards to identify these individual fatty acid isomers.

were increased in milk fat. The *trans*-11 18:1 and *cis*-9, *trans*-11 18:2 had not reached constant concentrations in milk fat by d 3 of the fatty acid infusion. In contrast, the increase in *trans*-12 18:1 and its desaturase product (*cis*-9, *trans*-12 18:2) approached maximum concentrations in milk fat over the first 36 h, and these were maintained for the remaining 36 h of the abomasal infusion.

Estimates of *trans*-isomer recovery in milk fat components are preliminary because steady-state concentrations cannot be ensured during short-term infusions. This was especially evident for *trans*-11 18:1 and its desaturase product, *cis*-9, *trans*-11 CLA (Fig. 2). Using the mean of the pretreatment period plus the last 3 d of the post-treatment period for the baseline concentration, we made comparisons with d 3 of the fatty acid infusion period. Changes in milk fat yields of *trans*-11 18:1 and *cis*-9, *trans*-11 CLA accounted for 40 ± 8% (mean ± SEM) of the abomasally infused *trans*-11 18:1, of which 31 ± 9% was due to an increase in *cis*-9, *trans*-11 CLA. An average of 64 ± 9% of the abomasally infused *trans*-12 18:1 was accounted for by milk fat changes in yields of *trans*-12 18:1 and *cis*-9, *trans*-12 18:2, but in this case only 10 ± 2% of the incorporation was due to the increase in *cis*-9, *trans*-12 18:2 content of milk fat. Thus, slightly more than one half of the abomasally infused *trans* 18:1 fatty acids were accounted for by increases in related fatty acids in milk; in the case of *trans*-11 18:1, however, a substantially greater portion was represented by an increase in the specific fatty acid formed by the action of Δ^9-desaturase.

Experiment 2. To evaluate the quantitative importance of endogenous synthesis of CLA, we infused retinol palmitate and sterculic oil abomasally. In this case, we utilized a diet containing extruded full-fat soybeans (Table 1); this type of diet results in increased milk fat concentrations of *trans*-11 18:1 and CLA (Chouinard et al. 1997, Dhiman et al. 1999). Dry matter intake and milk yield were not influenced by treatments (**Table 2**).

TABLE 2

Performance of lactating dairy cows during abomasal infusion of retinol palmitate or sterculic oil

Variable	Treatment[1]			SEM	*P*
	Control	Retinol palmitate	Sterculic oil		
Dry matter intake, *kg/d*	23.8	24.3	24.3	0.4	0.64
Milk yield, *kg/d*	35.2	34.9	34.9	0.4	0.85
Fat					
%	3.49	3.19	3.42	0.09	0.08
kg/d	1.22[a]	1.10[b]	1.18[a]	0.03	0.03
Protein					
%	2.82[a]	2.72[b]	2.77[a]	0.02	0.02
kg/d	0.97[a]	0.92[b]	0.95[a]	0.01	0.01

[1] Treatments involved a 4-d abomasal infusion of water (control), retinol palmitate (4.8 g/d) or sterculic oil (9.7 g/d). Values (n = 3 cows) represent means of d 3 and 4 of infusion. Values in a row with different superscripts differ, $P < 0.05$.

Infusion with retinol palmitate resulted in minor decreases in milk content and yield of fat and protein (Table 2). Retinol palmitate had minimal effects on fatty acid composition of milk, although concentrations of CLA and palmitic acid were increased slightly (Table 3). However, yield of CLA was not affected, and the activity of Δ^9-desaturase appeared unaltered on the basis of the constant ratios of relevant saturated fatty acids and their Δ^9-desaturase products (Fig. 3).

Infusion of sterculic oil did not alter the content or yield of milk components, but distinct changes in the fatty acid composition of milk occurred. Consistent with an inhibition of Δ^9-desaturase, the ratios of fatty acid pairs dependent on this enzyme were altered. Milk fat ratios of 14:0 to 14:1, 16:0 to 16:1 and 18:0 to *cis*-9 18:1 were increased two- to threefold by treatment with sterculic oil (Fig. 3).

Infusion of sterculic oil also altered the relationship between *trans*-11 18:1 and *cis*-9, *trans*-11 CLA as shown by the temporal pattern over the infusion period (Fig. 4). By d 4 of

TABLE 3

Composition of milk fat during abomasal infusion of retinol palmitate and sterculic oil in lactating dairy cows

Fatty acid	Treatment[1]			SEM	*P*
	Control	Retinol palmitate	Sterculic oil		
	g/100 g fatty acids				
4:0	5.38[ab]	5.05[b]	5.61[a]	0.12	0.02
6:0	2.54	2.53	2.60	0.03	0.24
8:0	1.31	1.33	1.29	0.02	0.34
10:0	2.49	2.66	2.66	0.06	0.14
12:0	2.63	2.84	2.72	0.07	0.13
14:0	9.10[c]	9.83[b]	10.49[a]	0.22	0.004
14:1[2]	0.72[a]	0.79[a]	0.22[b]	0.04	0.001
15:0	0.88[b]	0.95[a]	0.98[a]	0.02	0.004
16:0	27.74[b]	29.80[a]	30.51[a]	0.43	0.002
16:1[3]	1.24[a]	1.25[a]	0.48[b]	0.07	0.001
17:0	0.52	0.51	0.51	0.02	0.96
18:0	12.67[b]	11.40[b]	19.08[a]	0.47	0.001
18:1, *cis*[4]	22.06[a]	20.44[a]	12.25[b]	0.66	0.001
18:1, *trans*[5]	3.64[b]	3.54[b]	4.09[a]	0.10	0.007
CLA[6]	0.42[b]	0.50[a]	0.25[c]	0.03	0.001
18:2[7]	2.69	2.71	2.64	0.04	0.34
18:3[8]	0.39	0.43	0.41	0.01	0.10
Others	3.58[a]	3.44[a]	3.21[b]	0.05	0.001

[1] Treatments involved a 4 d abomasal infusion of water (control), retinol palmitate (4.8 g/d) and sterculic oil (9.7 g/d). Values (n = 3 cows) represent average of d 3 and 4 of infusion. Values in a row with different superscripts differ, $P < 0.05$.

[2] Identification based on myristoleic acid. Procedure does not preclude the presence of other 14:1 isomers.

[3] Identification based on palmitoleic acid. Procedure does not preclude the presence of other 16:1 isomers.

[4] Identification based on oleic acid. Procedure does not preclude the presence of other 18:1 isomers.

[5] Identification based on *trans*-11 and *trans*-12 18:1 isomers. Procedure does not preclude the presence of other *trans* isomers.

[6] Conjugated linoleic acid. Identification based on *cis*-9, *trans*-11 CLA. Procedure allows for identification of 8/10, 9/11, 10/12 and 11/13 CLA isomers (*cis/trans* or *trans/cis*).

[7] Identification based on linoleic acid. Procedure does not preclude the presence of other 18:2 isomers.

[8] Identification based on linolenic acid. Procedure does not preclude the presence of other 18:3 isomers.

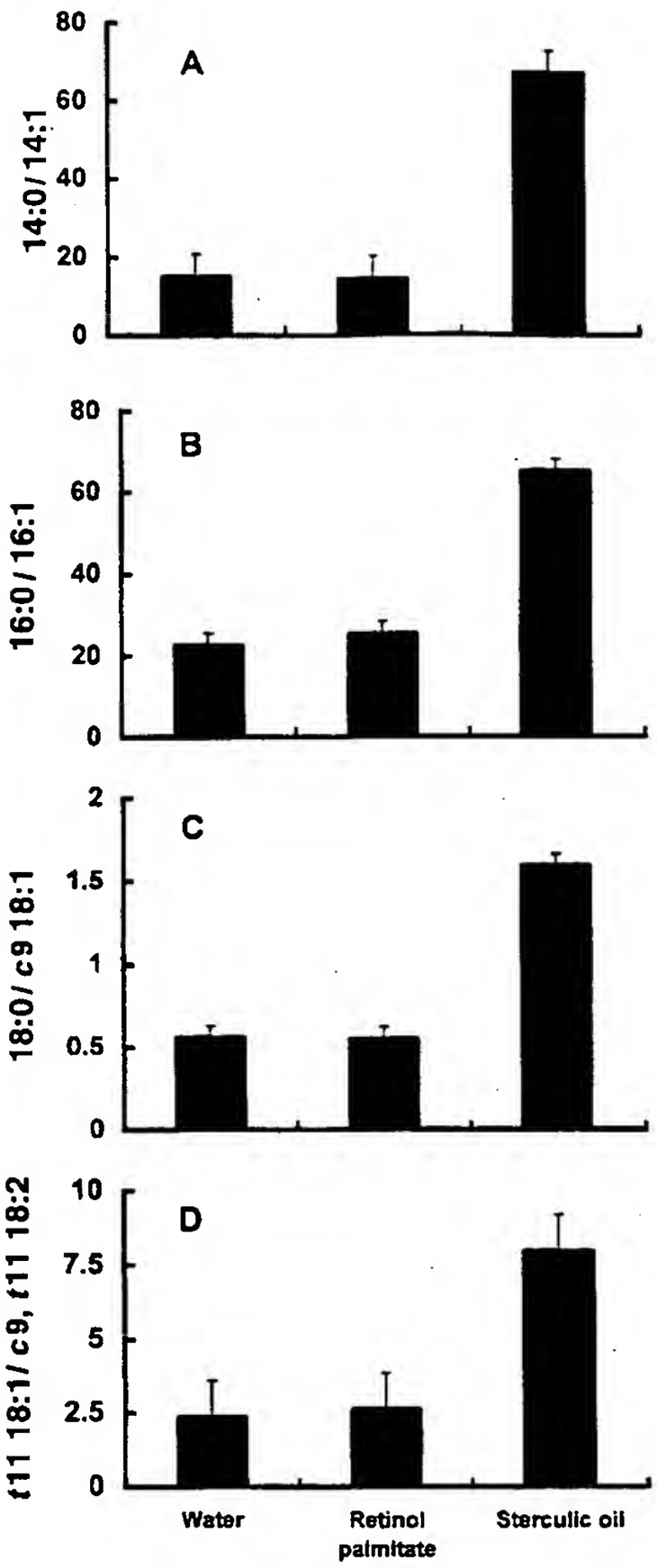

FIGURE 3 Ratio of the fatty acids to their Δ^9-desaturated products in milk fat of lactating cows (n = 3) receiving abomasal infusions of water, retinol palmitate and sterculic oil. Ratios are based on fatty acid mean values (± SEM) of d 3 and 4 of the treatment period. The analytical methods involved the use of pure standards to identify these individual fatty acid isomers. In all cases, sterculic oil treatment differed from the other two treatments ($P < 0.001$).

infusion, the ratio of *trans*-11 18:1 to CLA was increased twofold (Fig. 3), and the concentration and yield of CLA in milk fat were reduced 45%. A similar temporal pattern was also observed for concentration changes of the other desaturase pairs, i.e., 14:0 vs. 14:1, 16:0 vs. 16:1 and 18:0 vs. *cis*-9 18:1 (data not presented).

DISCUSSION

Conjugated linoleic acid is found predominantly in food products derived from ruminants, and it has been generally assumed that the CLA was of rumen origin [see for example, Chin et al. (1992), Parodi (1997 and 1999)]. Indeed, CLA is an intermediate in the biohydrogenation of linoleic acid by rumen bacteria, and the CLA content of ruminant fats can be increased by formulating diets that contain more linoleic acid (Dhiman et al. 1999, Kelly et al. 1998). However, kinetic studies of rumen biohydrogenation of linoleic acid to stearic acid have shown that *cis*-9, *trans*-11 CLA is a transient intermediate, whereas *trans*-11 18:1 is the intermediate that accumulates (Harfoot and Hazlewood 1988, Keeney 1970). Furthermore, dietary addition of plant oils containing α-linolenic acid also increase the CLA content of ruminant fat, and intermediates in its pathway of biohydrogenation include *trans*-11 18:1 but not CLA [see review by Griinari and Bauman (1999)]. Accordingly, we hypothesized that the CLA in ruminant tissues originated in part from endogenous synthesis.

Our initial experiment examined whether lactating cows could produce CLA from *trans*-11 18:1. Results clearly demonstrated that endogenous synthesis occurred. By d 3 of abomasal infusion of *trans*-11 18:1 (12.5 g/d), milk fat content of *cis*-9, *trans*-11 CLA had increased by 31%. The CLA concentration in milk fat had not reached a plateau by d 3 of infusion, indicating that studies of longer duration will be required to allow for definitive estimates of transfer efficiency, and these should also involve a range of *trans*-11 18:1 doses. Nevertheless, by d 3 of infusion, the increase in CLA concentration in milk fat accounted for 12% of the abomasally infused *trans*-11 18:1. The infused *trans*-octadecenoic acids consisted of a mixture of *trans*-11 18:1 and *trans*-12 18:1. Thus, *trans*-12 18:1 also provided a test for endogenous synthesis by Δ^9-desaturase with the product of the reaction being *cis*-9, *trans*-12 18:2. We observed the appearance of *cis*-9, *trans*-12 18:2 in milk fat during the infusion period, although the amount was substantially less than observed for conversion of *trans*-11 18:1 to *cis*-9, *trans*-11 CLA (Fig. 2).

The precursor for the endogenous synthesis of CLA in ruminants would be *trans*-11 18:1, which originates in the rumen from incomplete biohydrogenation of PUFA. Several studies have demonstrated that substantial amounts of *trans*-18:1 fatty acids (60–300 g/d) reach the duodenum in lactating cows (Kalscheur et al. 1997a and 1997b, Wonsil et al. 1994). Other investigations have established that *trans* fatty acids were absorbed efficiently from the digestive tract and utilized by different ruminant tissues, including the mammary gland (Bickerstaffe et al. 1972, Thompson and Christie 1991). Methods used in the above investigations did not allow for

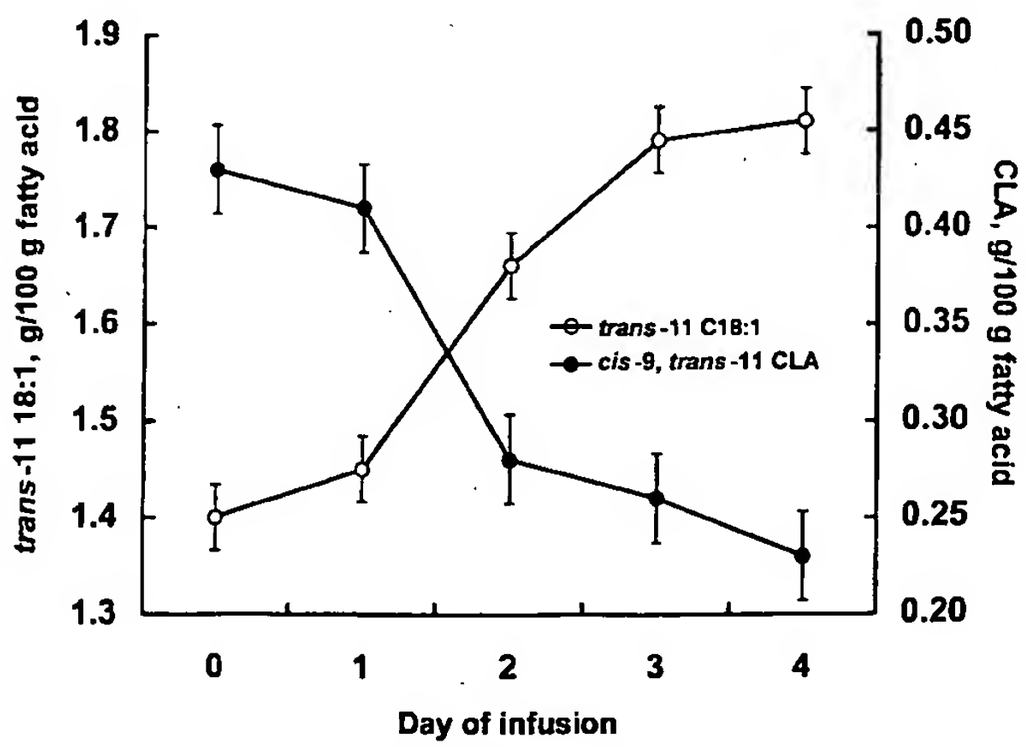

FIGURE 4 Temporal pattern of milk fat content of *trans*-11 18:1 and *cis*-9, *trans*-11 conjugated linoleic acid (CLA) in lactating cows (n = 3) before treatment (d 0) and during the 4 d of abomasal infusion of sterculic oil (9.7 g/d). Bars for each data point indicate SEM. The analytical methods involved the use of pure standards to identify these individual fatty acids.

separation of specific *trans*-18:1 isomers, but *trans*-11 has been shown to be the major *trans* octadecenoic acid isomer produced by rumen biohydrogenation under typical dietary conditions (Griinari and Bauman 1999).

In lactating cows, *trans*-18:1 fatty acids have been proposed to cause an inhibition of milk fat synthesis (Davis and Brown 1970, Erdman, 1996). Consistent with this, a decrease in milk fat yield occurs when partially hydrogenated vegetable oils were infused abomasally (Erdman 1996) and increases in milk fat content of *trans*-18:1 were highly correlated with reductions in the fat content of milk across a wide range of diets (Griinari et al. 1998). However, this effect appears to be related to specific *trans* isomers. We observed that abomasal infusion of 25 g/d of an equal mixture of *trans*-11 18:1 and *trans*-12 18:1 had no effect on milk fat yield or content (Fig. 1). Similarly, Rindsig and Schultz (1974) observed no reduction in milk fat when 25 g/d of *trans*-9 18:1 was infused abomasally. Other *trans*-18:1 isomers have not been examined, but we have shown that dietary-induced reductions in milk fat yield were closely related to specific increases in milk fat content of *trans*-10 18:1 and *trans*-10, *cis*-12 CLA (Griinari et al. 1998 and 1999). We further demonstrated that a dramatic reduction in milk fat secretion occurs in dairy cows with abomasal infusion of as little as 10 g/d of *trans*-10, *cis*-12 CLA, whereas infusion of *cis*-9, *trans*-11 CLA had no effect on milk fat synthesis (Baumgard et al. 2000).

The oxidative reaction catalyzed by Δ^9-desaturase for endogenous synthesis of CLA involves cytochrome b_5, NADH(P)-cytochrome b_5 reductase and molecular O_2 (Ntambi 1999). Palmitoyl-CoA and stearoyl-CoA are the primary substrates for the microsomal enzyme (Enoch et al. 1976), but Δ^9-desaturase can also use the CoA esters of *trans* fatty acids, including *trans*-11 18:1 (Mahfouz et al. 1980, Pollard et al. 1980). In rodents, the enzyme was located predominantly in the liver (Ntambi 1995). In contrast, adipose tissue was the major site for Δ^9-desaturase in growing ruminants, and mammary gland the major tissue site in lactating ruminants (Kinsella 1972, Martin et al. 1999, St. John et al. 1991, Ward et al. 1998). Studies with rodents have demonstrated that hepatic mRNA levels and enzyme activity of Δ^9-desaturase are regulated by many factors, including physiologic state, diet and hormonal balance (Ntambi 1995 and 1999, Tocher et al. 1998). Investigations with ruminants are more limited. Martin et al. (1999) characterized the ontogeny of Δ^9-desaturase gene expression in adipose tissue of growing cattle, and Ward et al. (1998) demonstrated that the onset of lactation in sheep resulted in a dramatic increase in mRNA for Δ^9-desaturase in mammary tissue and a reciprocal reduction in adipose tissue.

Our second experiment evaluated the quantitative significance of endogenous synthesis in the production of CLA found in milk fat. For this objective, treatments were designed to alter tissue activity of Δ^9-desaturase. One treatment involved administration of retinol palmitate. Administration of retinol palmitate to mice dramatically increased hepatic expression of Δ^9-desaturase in both vitamin A–deficient and normal mice. Miller et al. (1997) demonstrated that liver desaturase mRNA levels were increased approximately three- and sevenfold in vitamin A–deficient and normal mice, respectively, when mice were fed 0.1% vitamin A in the diet. In our study, this treatment resulted in a significant increase in the concentration of CLA, but had no effect on the yield of CLA in milk fat. Overall, effects of retinol palmitate were relatively minor (Tables 2 and 3), and this treatment did not alter the milk fat ratio for any of the fatty acid pairs related to Δ^9-desaturase activity (Fig. 3).

A second treatment involved abomasal infusion of sterculic oil. As in the work of Kai and Pryde (1982), the sterculic oil contained 55.9% sterculic acid (8-[2-octyl-1-cyclopropenyl] octanoic acid) and 6.3% malvalic acid (7-[2-octyl-1-cyclopropenyl] heptanoic acid), fatty acids with a cyclopropene ring at the 9–10 position. These two cyclopropenoid fatty acids are very specific and highly potent inhibitors of Δ^9-desaturase (Jeffcoat and Pollard 1977). We observed that infusion with sterculic oil resulted in decreased *cis*-9, *trans*-11 CLA concentration and a reciprocal increase in the *trans*-11 18:1 content of milk fat (Fig. 4). This clearly demonstrates the critical role of Δ^9-desaturase as a source of the CLA in milk fat. Similar dramatic shifts were observed for the milk fat content of other fatty acid pairs that are affected by desaturase activity, i.e., 14:0:14:1, 16:0:16:1 and 18:0:*cis*-9 18:1. Thus, our experiment also confirms the important role of Δ^9-desaturase in the production of oleic acid and provides the first evidence that this enzyme reaction is a major source of the myristoleic acid and palmitoleic acid found in milk fat.

Sterculic oil has been used previously to inhibit Δ^9-desaturase, generally to study the role of this enzyme in the conversion of stearic acid to oleic acid. Previous investigations have included rodents, chickens and other species [see for example, Fan et al. (1982) and Phelps et al. (1965)]. Investigations have also included lactating goats and cows; these single-animal studies have reported increases in the 18:0:18:1 ratio in milk fat when sterculic oil was given by abomasal infusion (Bickerstaffe and Johnson 1972, Porter 1984) or by dietary addition of a rumen-protected form (Cook et al. 1976). Our specific interest was to evaluate the importance of endogenous synthesis of CLA, and we observed a 45% reduction in the milk fat content with the sterculic oil treatment. Thus, under the dietary conditions of the present experiment, a minimum of one half of the CLA in milk fat was of endogenous origin involving Δ^9-desaturase.

The above estimate is a minimum based on the assumption that the sterculic oil dose inhibited Δ^9-desaturase completely. Complete inhibition is unlikely, but the extent of Δ^9-desaturase inhibition can be evaluated by comparing results from other fatty acid pairs that represent substrate:product ratios for the enzyme. A portion of the palmitoleic acid and oleic acid in milk fat could originate from mammary gland uptake of these fatty acids. However, comparison of 14:0 with 14:1 is ideal because 14:0 originates from mammary gland synthesis, and essentially the only source for myristoleic acid in milk fat is desaturation by Δ^9-desaturase. During the sterculic oil treatment, the secretion of 14:1 in milk fat was reduced to 30% of the control period, indicating that inhibition of Δ^9-desaturase was ~70%. Applying this adjustment to the relationship between *trans*-11 18:1 and CLA gives an estimate that ~64% of the CLA in milk fat originated via Δ^9-desaturase. This is a maximum estimate, which assumes that all of the 14:1 is *cis*-9 14:1 that originates from endogenous synthesis. In addition, the kinetics for sterculic acid and malvalic acid inhibition of Δ^9-desaturase have not been compared for different substrates, making this a limitation in extending inhibition estimates across substrates. Nevertheless, it is clear that endogenous synthesis via Δ^9-desaturase represents the major source of CLA in milk fat.

A close linear relationship between *trans*-11 18:1 fatty acid and CLA has been observed for milk fat in a number of studies and across a wide range of diets [see review by Griinari and Bauman (1999)]. This relationship has been generally attributed to a common source for these two fatty acids as intermediates in ruminal biohydrogenation. However, our studies demonstrate that the close relationship between *trans*-11 18:1 and CLA in milk fat is related to the formation of *cis*-9, *trans*-11 CLA from *trans*-11 18:1 via Δ^9-desaturase. This close

relationship has also been observed over a wide range of *trans*-11 18:1 concentrations (Griinari and Bauman 1999), suggesting a high capacity for endogenous synthesis of CLA. This is an important consideration in developing feeding strategies for the production of CLA-enriched milk. The focus should be on ruminal formation of *trans*-11 18:1 rather than CLA. In practical terms, this means that the most feasible options to enhance milk fat CLA concentrations may be to feed supplements containing *trans*-11 18:1 or dietary management of rumen biohydrogenation to increase the formation of *trans*-11 18:1 (Griinari and Bauman 1999).

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The role of Δ^9-desaturase in the production of *cis*-9, *trans*-11 CLA☆

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Abstract

Biomedical studies with animal models have demonstrated that *cis*-9, *trans*-11 conjugated linoleic acid (CLA), the predominant isomer found in milk fat from dairy cows, has anticarcinogenic effects. We recently demonstrated endogenous synthesis of *cis*-9, *trans*-11 CLA from ruminally derived *trans*-11 C18:1 by Δ^9-desaturase in lactating dairy cows. The present study further examined endogenous synthesis of *cis*-9, *trans*-11 CLA and quantified its importance by increasing substrate supply using partially hydrogenated vegetable oil (PHVO) as a source of *trans*-11 C18:1 and blocking endogenous synthesis using sterculic oil (SO) as a source of cyclopropene fatty acids which specifically inhibit Δ^9-desaturase. Four cows were abomasally infused with 1) control, 2) PHVO, 3) SO, and 4) PHVO+SO in a 4 x 4 Latin square design. With infusion of PHVO, *cis*-9, *trans*-11 CLA was increased by 17% in milk fat. Consistent with inhibition of desaturase, SO treatments increased milk fat ratios for the fatty acid pairs effected by Δ^9-desaturase, C14:0/*cis*-9 C14:1, C16:0/*cis*-9 C16:1, and C18:0/*cis*-9 C18:1. The role of endogenous synthesis of CLA was evident from the 60–65% reduction in *cis*-9, *trans*-11 CLA which occurred in milk fat with SO treatments. *cis*-9 C14:1 originates from desaturation of C14:0 by Δ^9-desaturase and can be used to estimate the extent of SO inhibition of Δ^9-desaturase. When this correction factor was applied, endogenous synthesis was estimated to account for 78% of the total *cis*-9, *trans*-11 CLA in milk fat. Thus, endogenous synthesis was the major source of *cis*-9, *trans*-11 CLA in milk fat of lactating cows.

Keywords: Δ^9-Desaturase; Trans fatty acid; Conjugated linoleic acid; Milk fat; Sterculic acid; Cyclopropene fatty acids

1. Introduction

Functional foods contain dietary components that have beneficial properties beyond their traditional nutrient value [1]. Conjugated linoleic acid (CLA) represents a functional food component found in dairy products. CLA is a collective term for isomers of linoleic acid with conjugated double bonds in several positions and conformations. The predominant CLA found in milk fat, the *cis*-9, *trans*-11 isomer, has been shown to be anticarcinogenic in animal models [2]. Butter containing significantly increased CLA concentrations has also been shown to be anticarcinogenic in a rat mammary cancer model [3].

Ruminal biohydrogenation of linoleic acid produces *cis*-9, *trans*-11 CLA as the first intermediate and it has been generally assumed that the CLA in milk fat had escaped complete biohydrogenation in the rumen [4]. However, we recently demonstrated endogenous synthesis of *cis*-9, *trans*-11 CLA in lactating dairy cows [5]. This pathway involves Δ^9-desaturase and the desaturation of ruminally derived *trans*-11 C18:1, the second intermediate of linoleic acid biohydrogenation. Others have also shown an endogenous synthesis of *cis*-9, *trans*-11 CLA in mice [6], rats [3], and humans [7,8] based on the increase in CLA observed with diets supplemented with *trans*-11 C18:1. The final step in rumen biohydrogenation of linoleic acid is the hydrogenation of *trans*-11 C18:1 to C18:0.

Our objective was to further examine endogenous synthesis of *cis*-9, *trans*-11 CLA by lactating dairy cows and quantify its importance by enhancing and inhibiting endog-

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Table 1
Ingredient and chemical composition of the experimental diet

Composition	Content
Ingredient (g/100 g dry matter)	
Chopped alfalfa hay	45.08
Ground corn	22.82
Soybean meal	7.04
Extruded soybeans	8.36
Citrus pulp	9.89
Whole cottonseed	4.40
Limestone	0.71
Dicalcium phosphate	0.69
Sodium bicarbonate	0.53
Magnesium oxide	0.16
Mineral-vitamin mix[1]	0.33
Chemical Analysis[2]	
Dry matter (g/100 g)	90.1
Crude protein (g/100 g dry matter)	16.4
Crude fat (g/100 g dry matter)	4.73
Neutral detergent fiber (g/100 g dry matter)	41.2
Acid detergent fiber (g/100 g dry matter)	31.2
Net energy for lactation (MJ/kg dry matter)	6.63

[1] Contained 385 g NaCl and 615 g of a trace mineral and vitamin mix per kg of supplement. Trace mineral and vitamin mix contained (g/kg mix) Mn, 1.1; Zn 1.4; Fe, 0.50; Cu, 0.25; I, 0.027; Co, 0.024; Se, 0.007; retinyl acetate, 0.258; cholecalciferol, 0.007; and *dl*-α-tocopheryl acetate, 2.56.
[2] Analyses were by Dairy One, Ithaca, NY.

enous synthesis. For the former we increased the substrate for endogenous synthesis using partially hydrogenated vegetable oil (PHVO) as a source of *trans*-11 C18:1. For the latter, we blocked endogenous synthesis using sterculic oil (SO) as a source of cyclopropene fatty acids which specifically inhibit Δ^9-desaturase [9].

2. Materials and methods

2.1. Animals and experimental design

All procedures using animals were approved by the Cornell University Institutional Animal Care and Use Committee. Four lactating multiparous, Holstein cows (115 ± 9 days in milk, mean ± SD) fitted with rumen cannulae were fed a total mixed ration (Table 1) formulated using the Cornell Net Carbohydrate and Protein System [10] to meet or exceed requirements [11]. Cows were fed *ad libitum* with fresh feed offered at 0600 and 1800 daily.

The experimental design was a 4 x 4 Latin square experiment with four day treatment periods and a six day interval between periods. Treatments were 1) control, 2) PHVO, 3) SO, and 4) PHVO+SO. The partially hydrogenated vegetable oil, obtained from Raisio Chemicals (Raisio, Finland), was solid at room temperature. For infusion (250 g/day), it was melted, infused, and then chased with 180 mL warm water (60°C) to evacuate the infusion line of any remaining oil. Sterculic oil was obtained from the seeds of the *Sterculia foetida* tree. Trees under private ownership

Table 2
Components and fatty acid composition of *Sterculia foetida* seeds

Variable	Content
Seed components (g/100 g total seeds)	
Hull	57.6
Meat	42.4
Pulp	25.2
Oil	17.2
Oil composition (g/100 g fatty acids)	
16:0	22.95
18:1, cyclo[1]	6.33
18:0	1.87
18:1, *cis*-9	5.08
18:2, *cis*-9, *cis*-12	5.03
19:1, cyclo[1]	55.86
Other	2.88

[1] cyclo = presence of cyclopropene ring. C18:1, cyclo is 7-(2-octyl-1-cyclopropenyl) heptanoic acid and C19:1, cyclo is 8-(2-octyl-1-cyclopropenyl) octanoic acid.

in India were identified and seeds harvested and imported by permit from USDA Animal and Plant Health Inspection Service (APHIS). Seed hulls were removed and the meat was then crushed and extracted using diethyl ether [12]. The extracted oil represented approximately 17% of the seeds and 41% of the seed meat (weight basis; Table 2). Sterculic oil was prepared as an emulsion in skim milk to provide sufficient volume for accurate infusion. Emulsions were prepared as previously described using a microfluidizer [13] and the target sterculic oil concentration was 2%. The actual concentration was 1.76 ± 0.15% (mean ± SD) so that the 500 mL/d delivered approximately 8.8 g/day of sterculic oil. An equal volume of skim milk was infused for the control treatment. Treatment solutions were infused into the abomasum via polyvinyl chloride tubing (0.5 cm i.d.), which passed through the rumen cannula and sulcus omasi into the abomasum [14]. One fourth of the daily dose was infused every 6 hr.

Cows were milked twice daily at 0600 and 1800. At each milking, yield was recorded and milk sampled. One aliquot was stored at 4°C with a preservative tablet (bronopol tablet; D & F Control System, San Ramon, CA) until infrared analysis for fat and protein content (Dairy One, Ithaca, NY). A second aliquot was stored at -20°C until analyzed for fatty acid composition. Blood samples were taken from the coccygeal vein after the 1800 milking on days 0, 3 and 4 of infusion. Sodium heparin (100 U/ml of blood) was used to prevent coagulation. Plasma was harvested (2,300 x *g*, 15 min at 4°C) and stored at -20°C until analyzed for fatty acid composition.

2.2. Analyses

For fatty acid analysis of milk, lipids were extracted using the method of Hara and Radin [15] as modified by Chouinard et al. [14] Fatty acid methyl esters were prepared

by transesterification with sodium methoxide [16] as adapted by Chouinard et al. [13] The fatty acids in SO were transmethylated by the same procedure as used for milk fat.

For fatty acid analysis of plasma, lipids were extracted using the method of Hara and Radin [15] with modifications. To 2.0 mL plasma was added 3.0 mL of hexane-isopropanol solution (3:2, v/v) followed by 2.0 mL sodium sulfate solution (67 g/L). The upper phase was removed and dried over 1.0 g anhydrous sodium sulfate. The solution was then transferred again and taken to dryness under a continuous stream of nitrogen. Plasma lipids were transmethylated with sodium methoxide according to the method of Christie [16]. Briefly, hexane (0.5 mL) was added to the extracted plasma lipids followed by 40 μL methyl acetate. After the mixture was vortexed, 40 μl methylation reagent (1.75 mL methanol: 0.4 mL of 5.4 M sodium methoxide) was added. The mixture was vortexed and allowed to react at room temperature for 24 hr, then 60 μL of termination reagent (1.0 g oxalic acid in 30 mL diethyl ether) were added. A few grains of calcium chloride were added to remove methanol. The solution containing the fatty acid methyl esters was removed for subsequent analysis.

Fatty acids in PHVO were methylated using trimethylsilyldiazomethane according to Hashimoto et al. [17] with modifications. To the fatty acids (40 mg), 1.6 mL hexane and 0.4 mL methanol were added. One mL of 30 mM trimethylsilyldiazomethane in hexane was added and allowed to stand at room temperature for 30 min. The reaction was stopped with 5 drops acetic acid. After the addition of 5.0 mL water, the hexane phase was dried over 1.0 g sodium sulfate and then transferred again and taken to dryness under a continuous stream of nitrogen. The fatty acid methyl esters were then dissolved in 2.0 mL hexane for analysis.

Fatty acid methyl esters from milk fat and sterculic oil were quantified by gas chromatography (Hewlett Packard GCD system HP G1800 A; Avondale, PA) equipped with a CP-Sil 88 column (100 m x 0.25 mm i.d. with 0.20 μm film thickness; Chrompack, The Netherlands). The analyses involved a programmed run with temperature ramps. The oven temperature was initially 50°C for 1 min then ramped to 160°C at 5°C/min and held for 42 min. The temperature was then ramped again at 5°C/min to 190°C and held for 22 min. Injector and detector temperatures were maintained at 250°C, the helium carrier gas flow rate was 1 ml/min, and the split ratio was 100:1.

Fatty acid methyl esters from plasma and PHVO were quantified by gas chromatography (Hewlett Packard GC system 6890+) equipped with a flame ionization detector and equipped with a SP-2560 capillary column (100 m × 0.25 mm i.d. with 0.2 μm film thickness; Supelco Inc., Bellefonte, PA). The oven temperature was initially 160°C and held for 28 min. The oven temperature was then ramped at 5°/min to 220° and held for 25 min. Inlet and detector temperature were maintained at 250°C and the split ratio was 100:1. The hydrogen carrier gas flow rate was 1 mL/min. Hydrogen flow to the detector was 25 ml/min, air flow was 400 ml/min, and the nitrogen make-up gas flow was 45 ml/min.

Table 3
Fatty acid composition of the partially hydrogenated vegetable oil

Fatty acid	Content (g/100 g fatty acids)
12:0	1.18
14:0	0.61
16:0	11.70
18:0	5.61
18:1, *trans*-6 to 8	3.61
18:1, *trans*-9	12.80
18:1, *trans*-10	12.15
18:1, *trans*-11	9.40
18:1, *trans*-12	6.09
Other	36.85

For both gas chromatography systems, each peak was identified and quantified using pure methyl ester standards (Nu Chek Prep, Elysian, MN). A butter reference standard (CRM 164; Commission of the European Communities, Community Bureau of Reference, Brussels, Belgium) was used to determine recoveries and correction factors for individual fatty acids.

2.3. Statistics

Data were statistically analyzed as a 4 x 4 Latin square using the PROC Mixed procedure of SAS [18]. For milk fatty acids, the model included treatment, period, and cow. The model for plasma fatty acids included treatment, period, and cow with day 0 as a covariate. Significant differences between treatments were determined using a *t* test.

3. Results

Dietary supplements of PHVO and SO were abomasally infused as a convenient experimental method to avoid ruminal biohydrogenation. PHVO provided a supply of *trans*-11 C18:1 as substrate for Δ^9-desaturase synthesis of *cis*-9, *trans*-11 CLA. The PHVO supplement contained approximately 44% *trans* fatty acids with *trans*-11 C18:1 accounting for 9.4% of the total fatty acids (Table 3). Thus, the infusion supplied approximately 23.5 g/day of *trans*-11 C18:1. The ability of SO to block endogenous synthesis of CLA via inhibition of Δ^9-desaturase was due to the cyclopropene fatty acids, sterculic acid (C19:1) and malvalic acid (C18:1). In the present study, these two cyclopropene fatty acids, comprised 62.2% of the sterculic oil (Table 2).

Milk yield and dry matter intake were unaffected by experimental treatments (Table 4). Similarly, milk protein yield was not affected, although small differences in milk protein content occurred (Table 4). In contrast, experimental treatments modestly reduced milk fat yield and content (8–17%).

Table 4
Performance of lactating dairy cows during abomasal infusion of partially hydrogenated vegetable oil (PHVO) and sterculic oil (SO)

Variable	Treatment				SEM	P^1
	Control	PHVO	SO	PHVO + SO		
Dry Matter Intake (kg/d)	27.3	25.1	26.3	26.7	0.6	0.10
Milk (kg/d)	39.8	37.8	38.6	38.4	0.7	0.27
Milk Protein						
kg/d	1.08	1.04	1.07	1.08	0.02	0.44
%	2.71[b]	2.75[bc]	2.78[ac]	2.83[a]	0.01	0.01
Milk Fat						
kg/d	1.33[a]	1.11[b]	1.17[b]	1.25[ab]	0.05	0.03
%	3.32[a]	2.90[b]	3.03[bc]	3.24[ac]	0.08	0.01

[1] Statistical probability of treatment differences. Values represent means for days 3 and 4 of the treatment period and means within row with different superscripts differ ($P < 0.05$).

Effects of treatment on the fatty acid composition of plasma and milk fat are presented in Tables 5 and 6. PHVO treatment resulted in the expected increase in *trans*-C18:1 fatty acids in plasma and milk fat. Specifically, *trans*-11 C18:1 content was increased by 46% in plasma and 39% in milk fat. Consistent with endogenous synthesis of CLA, PHVO infusion also increased *cis*-9, *trans*-11 CLA, and this was especially apparent in milk fat. Treatment with SO inhibited Δ^9-desaturase as evident by changes in fatty acid composition. For milk fatty acids, decreases of 84%, 59% and 46% were observed for *cis*-9 C14:1, *cis*-9 C16:1, and *cis*-9 C18:1, respectively. Consistent with inhibition of desaturase, SO treatment increased milk fat ratios for the fatty acid pairs effected by Δ^9-desaturase, C14:0/*cis*-9 C14:1, C16:0/*cis*-9 C16:1, and C18:0/*cis*-9 C18:1. Plasma concentrations of *cis*-9 C16:1 and *cis*-9 C18:1 were also reduced with SO treatment, although to a lesser extent. The role of endogenous synthesis of CLA was evident from the marked reduction in *cis*-9, *trans*-11 CLA and the 18% increase in *trans*-11 C18:1 which occurred in milk fat with SO treatment. The temporal pattern for *cis*-9, *trans*-11 CLA and *trans*-11 C18:1 further illustrated the reciprocal changes in these fatty acids when SO was infused (Fig. 1).

The PHVO+SO treatment gave mixed results in terms of changes in plasma and milk fatty acids. *trans* fatty acids were increased in a manner similar to the PHVO treatment whereas fatty acids related to Δ^9-desaturase were decreased similar to the SO treatment (Tables 5 and 6). However, there

Table 5
Composition of plasma lipids during abomasal infusion of partially hydrogenated vegetable oil (PHVO) and sterculic oil (SO) in lactating dairy cows

Fatty Acid	Treatment				SEM	P^1
	Control	PHVO	SO	PHVO + SO		
Content (g/100 g fatty acids)						
15:0	0.51[b]	0.53[a]	0.53[a]	0.54[a]	<0.01	0.01
16:0	8.42[b]	8.78[a]	8.33[b]	8.28[b]	0.11	0.02
16:1, *cis*-9	0.23[a]	0.23[a]	0.21[b]	0.21[b]	<0.01	0.01
17:0	0.62[a]	0.58[b]	0.62[a]	0.58[b]	0.01	0.02
18:0	16.08[a]	15.30[b]	16.22[a]	14.98[b]	0.30	0.05
18:1, *trans*-6 to 8	0.07[b]	0.12[a]	0.08[b]	0.12[a]	<0.01	0.01
18:1, *trans*-9	0.07[c]	0.31[a]	0.07[c]	0.29[b]	<0.01	0.01
18:1, *trans*-10	0.11[b]	0.24[a]	0.11[b]	0.23[a]	<0.01	0.01
18:1, *trans*-11	0.50[b]	0.73[a]	0.50[b]	0.73[a]	0.01	0.01
18:1, *trans*-12	0.36[b]	0.51[a]	0.34[b]	0.49[a]	<0.01	0.01
18:1, *cis*-9	2.93[b]	3.26[a]	2.68[d]	2.81[c]	0.04	0.01
18:2, *cis*-9, *cis*-12	46.34	45.61	46.67	46.94	0.50	0.29
18:3, *cis*-9, *cis*-12, *cis*-15	2.94	2.89	3.03	3.02	0.05	0.14
cis-9, *trans*-11 CLA	0.07[b]	0.08[a]	0.06[c]	0.06[c]	<0.01	0.01
20:4, *cis*-5, *cis*-8, *cis*-11, *cis*-14	2.10[a]	2.08[a]	2.04[ab]	2.00[b]	0.03	0.04
Others	18.65	18.76	18.51	18.72	0.30	0.49
Ratio						
16:0/*cis*-9 16:1	39.13	38.51	39.28	39.79	1.34	0.89
18:0/*cis*-9 18:1	5.51[b]	4.73[c]	6.09[a]	5.34[b]	0.07	0.01
trans-11 18:1/*cis*-9, *trans*-11 CLA	7.49[d]	10.07[b]	8.76[c]	12.20[a]	0.40	0.01

[1] Statistical probability of treatment differences. Values represent means for the last two milkings of the treatment period and means within row with different superscripts differ ($P < 0.05$).

Table 6
Composition of milk fat during abomasal infusion of partially hydrogenated vegetable oil (PHVO) and sterculic oil (SO) in lactating dairy cows

Fatty Acid	Treatment				SEM	P^1
	Control	PHVO	SO	PHVO + SO		
Content (g/100 g fatty acids)						
4:0	5.67[a]	4.90[b]	5.46[a]	4.95[b]	0.17	0.02
6:0	2.40[ab]	2.24[b]	2.67[a]	2.21[b]	0.10	0.01
8:0	1.20[ab]	1.08[b]	1.30[a]	1.04[b]	0.06	0.01
10:0	2.45[a]	1.85[c]	2.37[a]	2.15[b]	0.04	0.01
12:0	2.56[a]	2.12[c]	2.35[b]	2.31[b]	0.03	0.01
14:0	9.56[a]	7.96[c]	9.74[a]	9.21[b]	0.10	0.01
14:1, *cis*-9	0.55[a]	0.50[a]	0.09[b]	0.11[b]	0.02	0.01
15:0	0.81[a]	0.72[c]	0.78[b]	0.73[c]	<0.01	0.01
16:0	26.26[a]	23.25[b]	27.24[a]	24.08[b]	0.29	0.01
16:1, *cis*-9	0.73[a]	0.75[a]	0.30[b]	0.26[b]	0.03	0.01
17:0	0.47[b]	0.43[c]	0.52[a]	0.47[b]	0.01	0.01
18:0	14.30[c]	13.62[c]	23.07[a]	20.15[b]	0.35	0.01
18:1, *trans*-6 to 8	0.39[d]	0.78[b]	0.49[c]	0.89[a]	0.03	0.01
18:1, *trans*-9	0.28[c]	2.12[a]	0.29[c]	1.96[b]	0.03	0.01
18:1, *trans*-10	0.62[b]	2.09[a]	0.69[b]	2.16[a]	0.03	0.01
18:1, *trans*-11	2.18[d]	3.03[b]	2.57[c]	3.81[a]	0.05	0.01
18:1, *trans*-12	0.63[d]	1.37[b]	0.74[c]	1.51[a]	0.02	0.01
18:1, *cis*-9	20.66[a]	21.22[a]	11.21[c]	12.14[b]	0.29	0.01
18:2, *cis*-9, *cis*-12	4.04[b]	4.32[a]	4.02[b]	4.37[a]	0.08	0.01
18:3, *cis*-9, *cis*-12, *cis*-15	0.50	0.49	0.46	0.49	0.01	0.09
cis-9, *trans*-11 CLA	0.65[b]	0.76[a]	0.23[d]	0.30[c]	0.02	0.01
Others	1.79	2.30	2.18	1.86	0.06	0.01
Ratio						
14:0/*cis*-9 14:1	16.82[c]	16.26[c]	111.65[a]	85.82[b]	4.76	0.01
16:0/*cis*-9 16:1	65.32[b]	31.55[b]	118.84[a]	115.20[a]	10.74	0.01
18:0/*cis*-9 18:1	0.73[c]	0.64[c]	2.06[a]	1.66[b]	0.03	0.01
trans-11 18:1/*cis*-9, *trans*-11 CLA	3.59[c]	3.99[c]	11.63[b]	13.08[a]	0.39	0.01

[1] Statistical probability of treatment differences. Values represent means for the last two milkings of the treatment period and means within row with different superscripts differ ($P < 0.05$).

are subtle differences in fatty acids between treatments which are graphically illustrated in Figures 1 and 2. With infusion of PHVO+SO, *trans*-11 C18:1 was greater than observed for the PHVO treatment. By inhibiting desaturase, the conversion of exogenous *trans*-11 C18:1 fatty acids to *cis*-9, *trans*-11 CLA was markedly reduced, resulting in the observed increase over the PHVO treatment. However, the inhibition by sterculic oil was incomplete as indicated by the milk fat content of *cis*-9, *trans*-11 CLA which was greater during the PHVO+SO treatment than observed for the SO treatment (Fig. 1). Thus, the supply of *trans*-11 C18:1 and incomplete inhibition of Δ^9-desaturase resulted in greater *cis*-9, *trans*-11 CLA with the PHVO+SO treatment.

Δ^9-Desaturase adds a double bond across carbons 9 and 10. *trans*-8, *trans*-9, and *trans*-10 C18:1 fatty acids all have a pre-existing double bond at the site where Δ^9-desaturase would act. However, other measured *trans* fatty acids do not have this complication and consistent with a desaturation of these fatty acids, the SO treatment increased the milk fat content of *trans*-12 C18:1 and the coeluting *trans*-6–8 C18:1 in a manner similar to *trans*-11 C18:1 (Fig. 1 and *2*). In contrast, the milk fat content of *trans*-9 C18:1 and *trans*-10 C18:1 were identical for PHVO and PHVO+SO treatments consistent with the inability of these fatty acids to serve as substrates for Δ^9-desaturase.

4. Discussion

There is interest in increasing the concentration of *cis*-9, *trans*-11 CLA in milk fat. This stems from recent findings that *cis*-9, *trans*-11 CLA is the predominant isomer found in milk fat from dairy cows and it has anticarcinogenic effects in biomedical studies with animal models [2,3]. Diet has a major effect on milk fat concentration of *cis*-9, *trans*-11 CLA and dietary conditions which increase milk fat content have been described [19]. The *cis*-9, *trans*-11 CLA in milk fat has been assumed to represent *cis*-9, *trans*-11 CLA produced in the rumen by biohydrogenation of dietary polyunsaturated fatty acids [4,20]. While ruminal production of *cis*-9, *trans*-11 CLA contributes to milk fat CLA, we demonstrated that endogenous synthesis of *cis*-9, *trans*-11 CLA also makes a significant contribution [5]. Endogenous synthesis of *cis*-9, *trans*-11 CLA involves the enzyme Δ^9-desaturase and the substrate is *trans*-11 C18:1, another intermediate formed in the ruminal biohydrogenation of polyunsaturated C18-fatty acids. In order to quantify the

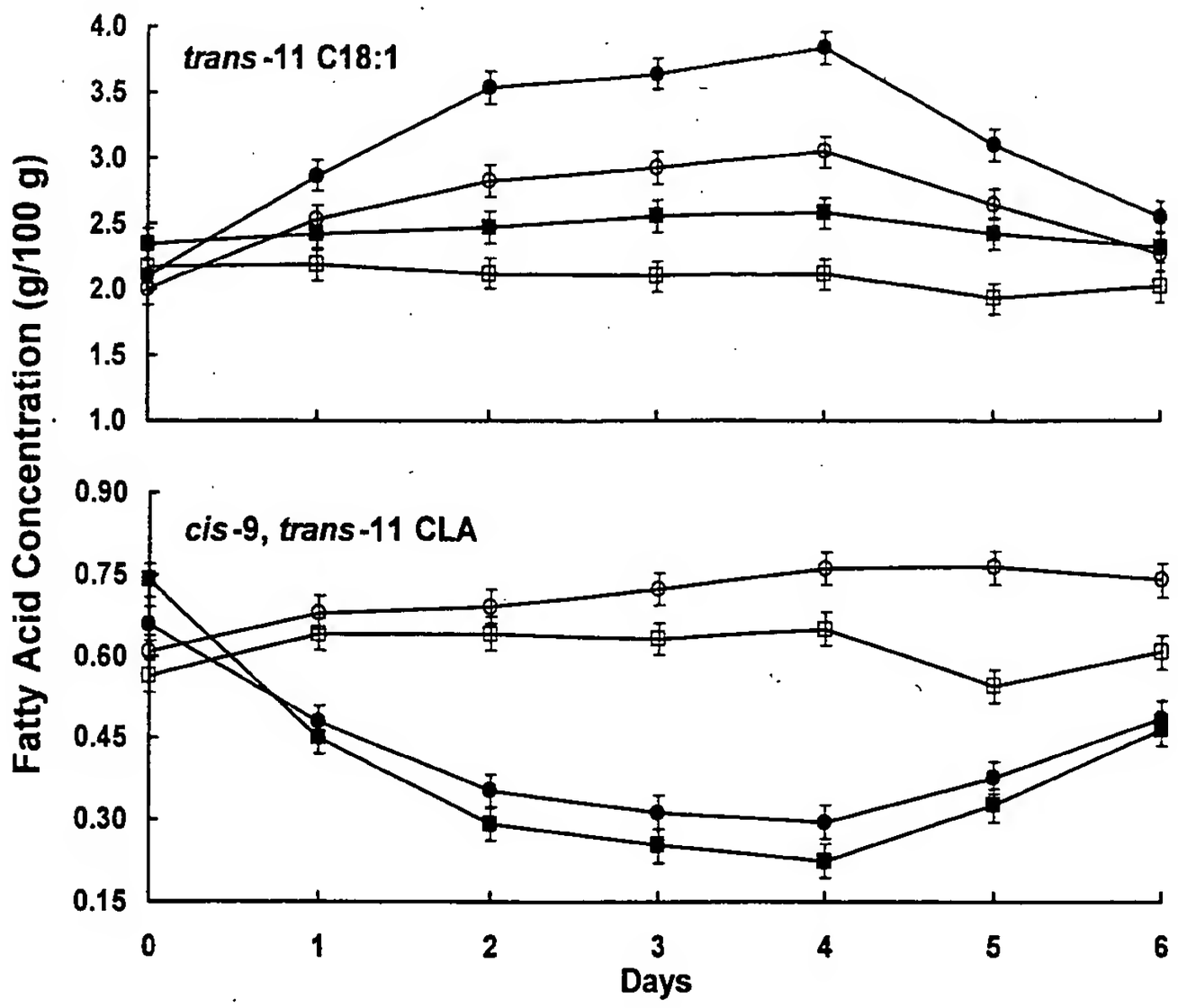

Fig. 1. Temporal pattern of *trans*-11 C18:1 (upper panel) and *cis*-9, *trans*-11 CLA (lower panel) in milk fat of lactating dairy cows receiving abomasal infusion of partially hydrogenated vegetable oil (250 g/day), sterculic oil (8.8 g/day), or both. Treatment commenced on day 1 and lasted through day 4. Values represent the mean of 4 cows with 2 milkings per day and bars on each data point indicate SE. Open squares = control, closed squares = sterculic oil, open circles = partially hydrogenated vegetable oil, and closed circles = partially hydrogenated vegetable oil plus sterculic oil.

relative contribution of endogenous synthesis, the present study supplied *trans*-11 C18:1 as exogenous substrate for desaturation to *cis*-9, *trans*-11 CLA as well as cyclopropene fatty acids to inhibit Δ^9-desaturase.

PHVO served as the source of *trans*-11 C18:1 and SO provided the cyclopropene fatty acids. Abomasal infusion of PHVO increased the milk fat content of CLA and *trans*-11 C18:1 by 17% and 39%, respectively. Likewise, inhibition of Δ^9-desaturase with SO dramatically reduced milk fat CLA. The reduction was 65% for SO versus control treatments and 61% for the comparison of PHVO+SO and PHVO treatments. Therefore, the results indicate endogenous synthesis is of major importance in supplying the CLA found in milk fat. Reductions observed with SO represent a minimum estimate of endogenous synthesis because complete inhibition of Δ^9-desaturase was not achieved. This is indicated by the PHVO+SO treatment that yielded a milk fat content of *cis*-9, *trans*-11 CLA significantly greater than the SO treatment. Incomplete inhibition is also illustrated by the presence of *cis*-9 C14:1 in milk fat for the SO and PHVO+SO treatments. Myristic acid (C14:0) is predominantly synthesized *de novo* by the mammary gland and *cis*-9 C14:1 originates from desaturation of C14:0 by Δ^9-desaturase located in mammary epithelial cells [21]. Consequently, the magnitude of the SO-induced reduction in C14:1 provides an estimate of the inhibition of Δ^9-desaturase. When this correction factor was applied, estimated endogenous synthesis was 78% of the total *cis*-9, *trans*-11 CLA in milk fat. Thus, endogenous synthesis was the major source of *cis*-9, *trans*-11 CLA in milk fat of lactating cows. In a previous experiment [5], endogenous synthesis was estimated to account for 64% of the total *cis*-9, *trans*-11 CLA in milk fat.

Cyclopropene fatty acids are found in the oils of many plant seeds including seeds from tropical trees, but also common flowers [22]. In the case of animal feeds, cottonseed oil contains measurable quantities of cyclopropene fatty acids and they can negatively impact animal performance due to inhibition of Δ^9-desaturase [22]. For example, in chickens, cyclopropene fatty acids inhibit Δ^9-desaturase and yolks become hard following refrigeration due to increased saturation of the yolk fatty acids [22]. However, ruminants do not typically experience negative effects since cyclopropene fatty acids represent a minor portion of the total diet and ruminal biohydrogenation inactivates them [23]. The sterculic oil used in the present study contained sterculic acid and malvalic acid (Table 2), both of which have a cyclopropene ring involving carbon 9. Jeffcoat and Pollard [9] demonstrated that fatty acids with a cyclopropene ring in this position are very potent inhibitors of Δ^9-desaturase. Others have also used cyclopropene fatty acids to inhibit Δ^9-desaturase in lactating ruminants at doses similar to the present study [5,23–25].

Milk fat contains several pairs of fatty acids that reflect a substrate-product relationship for Δ^9-desaturase. Four of these pairs are easily measured and ratios of these fatty acid

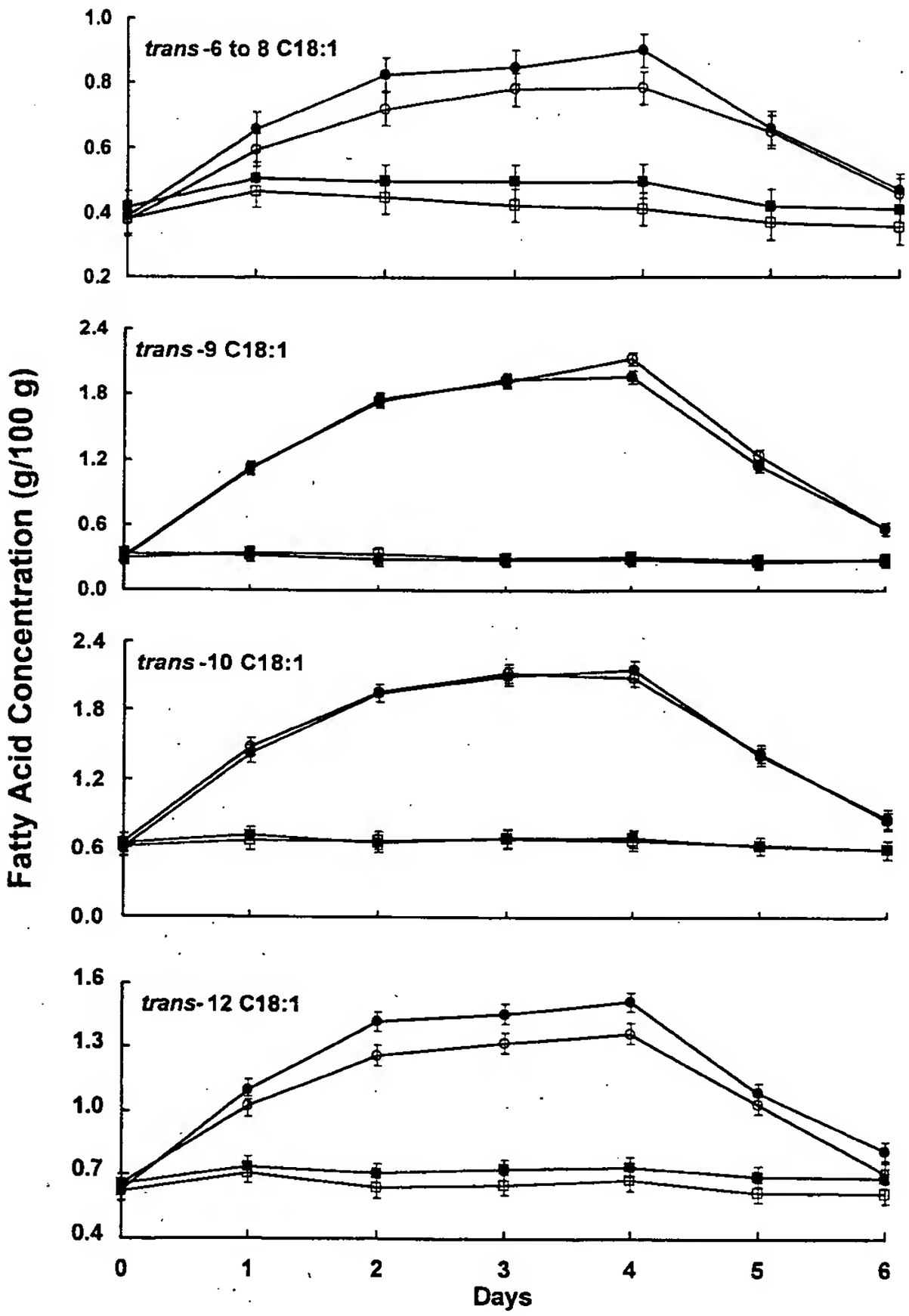

Fig. 2. Temporal pattern of *trans*-6–8 C18:1, *trans*-9 C18:1, *trans*-10 C18:1, and *trans*-12 C18:1 in milk fat of lactating dairy cows receiving abomasal infusion of partially hydrogenated vegetable oil (250 g/day), sterculic oil (8.8 g/day), or both. Treatment commenced on day 1 and lasted through day 4. Values represent the mean of 4 cows with 2 milkings per day and bars on each data point indicate SE. Open squares = control, closed squares = sterculic oil, open circles = partially hydrogenated vegetable oil, and closed circles = partially hydrogenated vegetable oil plus sterculic oil.

pairs were markedly altered when Δ^9-desaturase was inhibited with SO treatment (Table 6). Effects on the ratios of the Δ^9-desaturase fatty acid pairs were much smaller in plasma, although *cis*-9, *trans*-11 CLA and *cis*-9 C18:1 were reduced with SO treatment (Table 5). Thus, the mammary gland must be the major site of Δ^9-desaturase in lactating cows. A similar conclusion was reached by Bickerstaffe and Johnson [24] when they intravenously infused SO to a single goat. Studies comparing mRNA abundance [26] and activity [27–29] for Δ^9-desaturase have also demonstrated the mammary gland to be the major site for Δ^9-desaturase, although adipose tissue and intestine have detectable mRNA and/or enzyme activity [26,30,31]. Liver is a major site of Δ^9-desaturase in rodents [32] and this contrasts with ruminants. Relatively low abundance of Δ^9-desaturase mRNA has been observed in ruminant liver [26] and some studies have even failed to detect mRNA and enzyme activity in the liver [31,33].

The use of SO to inhibit Δ^9-desaturase also offers a unique opportunity to examine the role of this enzyme in the synthesis of milk fatty acids in general. Differences in milk fat content of *trans*-11 C18:1 between control versus SO treatments and PHVO versus PHVO+SO treatments reflect Δ^9-desaturase use of *trans*-11 C18:1 in the synthesis of *cis*-9, *trans*-11 CLA (Fig. 1). In contrast, milk fat content of *trans*-9 C18:1 and *trans*-10 C18:1 were not different for the same treatment comparisons because these fatty acids can not serve as substrates for Δ^9-desaturase. However, there were differences between control vs. SO and PHVO vs. PHVO+SO in milk fat content of *trans*-6–8 C18:1 and

trans-12 C18:1 indicating these fatty acids serve as substrates of Δ^9-desaturase. We previously demonstrated addition of a *cis*-9 bond to *trans*-12 C18:1 produces the *cis*-9, *trans*-12 C18:2 found in milk fat [5]. Differences in *trans*-6–8 C18:1 probably reflect use of *trans*-7 C18:1 to synthesize *trans*-7, *cis*-9 CLA, although methods used in the present study do not allow separation of this fatty acid. The presence of *trans*-7, *cis*-9 CLA in milk fat of ruminants has recently been reported by Yurawecz et al. [34] using more sophisticated analytical techniques.

Milk yield and milk protein were not altered by treatments, although milk fat yield was slightly reduced by SO and PHVO treatments. Bickerstaffe and Johnson [24] also observed a decrease in milk fat yield with SO. This may relate to the plasticity needs for milk fat triglycerides to be secreted, as the unsaturated fatty acids arising from Δ^9-desaturase would play an important role in establishing the physical characteristics of milk fat (see review by Chilliard et al. [35]). Others have also reported a decrease in milk fat yield when large doses of PHVO are infused (see review by Griinari and Bauman [4]). This apparently relates to the presence of specific fatty acids with a *trans*-10 double bond that inhibit milk fat synthesis, especially *de novo* synthesis.

Overall, results from the present study clearly demonstrate that endogenous synthesis is the major source of *cis*-9, *trans*-11 CLA in milk fat of lactating cows. The rumen is a source for a lesser portion of the *cis*-9, *trans*-11 CLA found in milk fat, but of greater importance is rumen production of *trans*-11 C18:1 which serves as the substrate for endogenous synthesis of CLA. Thus, the substantial variation which has been observed in milk fat content of CLA [19] is likely to be related to differences in ruminal production of *trans*-11 C18:1 as well as animal differences in Δ^9-desaturase activity. Interestingly, humans and other species are also capable of endogenous synthesis of CLA via Δ^9-desaturase with *trans*-11 C18:1 consumed in the diet serving as the substrate [3,6–8].

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United States Court of Appeals for the Federal Circuit

03-1480, -1481
(Interference No. 103,887)

DANIEL J. CAPON, ARTHUR WEISS, BRIAN A. IRVING,
MARGO R. ROBERTS, and KRISZTINA ZSEBO,

Appellants,

v.

ZELIG ESHHAR, DANIEL SCHINDLER, TOVA WAKS,
and GIDEON GROSS,

Cross-Appellants,

v.

JON DUDAS, Director of the Patent and Trademark Office,

Intervenor.

Steven B. Kelber, Piper Rudnick, LLP, of Washington, DC, argued for appellants.

Roger L. Browdy, Browdy and Neimark, P.L.L.C., of Washington, DC, argued for cross-appellants.

Mary L. Kelly, Associate Solicitor, Office of the Solicitor, United States Patent and Trademark Office, of Arlington, Virginia, argued for intervenor. With her on the brief were John M. Whealan, Solicitor and Stephen Walsh, Associate Solicitor.

Appealed from: United States Patent and Trademark Office Board of Patent Appeals and Interferences

United States Court of Appeals for the Federal Circuit

03-1480, -1481
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JON DUDAS,
Director of the Patent and Trademark Office,
Intervenor.

DECIDED: August 12, 2005

Before NEWMAN, MAYER,* and GAJARSA, Circuit Judges.

NEWMAN, Circuit Judge.

Both of the parties to a patent interference proceeding have appealed the decision of the Board of Patent Appeals and Interferences of the United States Patent and Trademark Office, wherein the Board held that the specification of neither party met the written description requirement of the patent statute. Capon v. Eshhar, Interf. No. 103,887

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* Haldane Robert Mayer vacated the position of Chief Judge on December 24, 2004.

(Bd. Pat. App. & Interf. Mar. 26, 2003). The Board dissolved the interference and cancelled all of the claims of both parties corresponding to the interference count. With this ruling, the Board terminated the proceeding and did not reach the question of priority of invention. We conclude that the Board erred in its application of the law of written description. The decision is vacated and the case is remanded to the Board for further proceedings.

BACKGROUND

Daniel J. Capon, Arthur Weiss, Brian A. Irving, Margo R. Roberts, and Krisztina Zsebo (collectively "Capon") and Zelig Eshhar, Daniel Schindler, Tova Waks, and Gideon Gross (collectively "Eshhar") were the parties to an interference proceeding between Capon's United States Patent No. 6,407,221 ("the '221 patent") entitled "Chimeric Chains for Receptor-Associated Signal Transduction Pathways" and Eshhar's patent application Serial No. 08/084,994 ("the '994 application") entitled "Chimeric Receptor Genes and Cells Transformed Therewith." Capon's Patent No. 5,359,046 ("the '046 patent"), parent of the '221 patent, was also included in the interference but was held expired for non-payment of a maintenance fee. The PTO included the '046 patent in its decision and in its argument of this appeal.[1]

A patent interference is an administrative proceeding pursuant to 35 U.S.C. §§102(g) and 135(a), conducted for the purpose of determining which of competing applicants is the first inventor of common subject matter. An interference is instituted after the separate

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1 Although Capon is designated as appellant and Eshhar as cross-appellant, both appealed the Board's decision. See Fed. R. App. P. 28(h). The Director of the PTO intervened to support the Board, and has fully participated in this appeal.

patent applications have been examined and found to contain patentable subject matter. Capon's patents had been examined and had issued before this interference was instituted, and Eshhar's application had been examined and allowed but a patent had not yet issued.

During an interference proceeding the Board is authorized to determine not only priority of invention but also to redetermine patentability. 35 U.S.C. §6(b). The question of patentability of the claims of both parties was raised *sua sponte* by an administrative patent judge during the preliminary proceedings. Thereafter the Board conducted an *inter partes* proceeding limited to this question, receiving evidence and argument. The Board then invalidated all of the claims that had been designated as corresponding to the count of the interference, viz., all of the claims of the Capon '221 patent, claims 5-8 of the Capon '046 patent, and claims 1-7, 9-20, and 23 of the Eshhar '994 application.

In accordance with the Administrative Procedure Act, the law as interpreted and applied by the agency receives plenary review on appeal, and the agency's factual findings are reviewed to determine whether they were arbitrary, capricious, or unsupported by substantial evidence in the administrative record. See 5 U.S.C. §706(2); Dickinson v. Zurko, 527 U.S. 150, 164-65 (1999); In re Gartside, 203 F.3d 1305, 1315 (Fed. Cir. 2000).

The Invention

A chimeric gene is an artificial gene that combines segments of DNA in a way that does not occur in nature. The '221 patent and '994 application are directed to the production of chimeric genes designed to enhance the immune response by providing cells with specific cell-surface antibodies in a form that can penetrate diseased sites, such as solid tumors, that were not previously reachable. The parties explain that their invention is

a way of endowing immune cells with antibody-type specificity, by combining known antigen-binding-domain producing DNA and known lymphocyte-receptor-protein producing DNA into a unitary gene that can express a unitary polypeptide chain. Eshhar summarized the problem to which the invention is directed:

> Antigen-specific effector lymphocytes, such as tumor-specific T cells, are very rare, individual-specific, limited in their recognition spectrum and difficult to obtain against most malignancies. Antibodies, on the other hand, are readily obtainable, more easily derived, have wider spectrum and are not individual-specific. The major problem of applying specific antibodies for cancer immunotherapy lies in the inability of sufficient amounts of monoclonal antibodies (mAb) to reach large areas within solid tumors.

Technical Paper Explaining Eshhar's Invention, at 6.

The inventions of Capon and Eshhar are the chimeric DNA that encodes single-chain chimeric proteins for expression on the surface of cells of the immune system, plus expression vectors and cells transformed by the chimeric DNA. The experts for both parties explain that the invention combines selected DNA segments that are both endogenous and nonendogenous to a cell of the immune system, whereby the nonendogenous segment encodes the single-chain variable ("scFv") domain of an antibody, and the endogenous segment encodes cytoplasmic, transmembrane, and extracellular domains of a lymphocyte signaling protein. They explain that the scFv domain combines the heavy and light variable ("Fv") domains of a natural antibody, and thus has the same specificity as a natural antibody. Linking this single chain domain to a lymphocyte signaling protein creates a chimeric scFv-receptor ("scFvR") gene which, upon transfection into a cell of the immune system, combines the specificity of an antibody with the tissue penetration, cytokine production, and target-cell destruction capability of a lymphocyte.

The parties point to the therapeutic potential if tumors can be infiltrated with specifically designed immune cells of appropriate anti-tumor specificity.

The Eshhar Claims

The Board held unpatentable the following claims of Eshhar's '994 application; these were all of the '994 claims that had been designated as corresponding to the count of the interference. Eshhar's claim 1 was the designated count.

1. A chimeric gene comprising

a first gene segment encoding a single-chain Fv domain (scFv) of a specific antibody and

a second gene segment encoding partially or entirely the transmembrane and cytoplasmic, and optionally the extracellular, domains of an endogenous protein

wherein said endogenous protein is expressed on the surface of cells of the immune system and triggers activation and/or proliferation of said cells,

which chimeric gene, upon transfection to said cells of the immune system, expresses said scFv domain and said domains of said endogenous protein in one single chain on the surface of the transfected cells such that the transfected cells are triggered to activate and/or proliferate and have MHC nonrestricted antibody-type specificity when said expressed scFV domain binds to its antigen.

2. A chimeric gene according to claim 1 wherein the second gene segment further comprises partially or entirely the extracellular domain of said endogenous protein.

3. A chimeric gene according to claim 1 wherein the first gene segment encodes the scFv domain of an antibody against tumor cells.

4. A chimeric gene according to claim 1 wherein the first gene segment encodes the scFv domain of an antibody against virus infected cells.

5. A chimeric gene according to claim 4 wherein the virus is HIV.

6. A chimeric gene according to claim 1 wherein the second gene segment encodes a lymphocyte receptor chain.

7. A chimeric gene according to claim 6 wherein the second gene segment encodes a chain of the T cell receptor.

9. A chimeric gene according to claim 7 wherein the second gene segment encodes the α, β, γ, or δ chain of the antigen-specific T cell receptor.

10. A chimeric gene according to claim 1 wherein the second gene segment encodes a polypeptide of the TCR/CD3 complex.

11. A chimeric gene according to claim 10 wherein the second gene segment encodes the zeta or eta isoform chain.

12. A chimeric gene according to claim 1 wherein the second gene segment encodes a subunit of the Fc receptor or IL-2 receptor.

13. A chimeric gene according to claim 12 wherein the second gene segment encodes a common subunit of IgE and IgG binding Fc receptors.

14. A chimeric gene according to claim 13 wherein said subunit is the gamma subunit.

15. A chimeric gene according to claim 13 wherein the second gene segment encodes the CD16α chain of the FcγRIII or FcγRII.

16. A chimeric gene according to claim 12 wherein the second gene segment encodes the α or β subunit of the IL-2 receptor.

17. An expression vector comprising a chimeric gene according to claim 1.

18. A cell of the immune system endowed with antibody specificity transformed with an expression vector according to claim 17.

19. A cell of the immune system endowed with antibody specificity comprising a chimeric gene according to claim 1.

20. A cell if the immune system according to claim 19 selected from the group consisting of a natural killer cell, a lymphokine activated killer cell, a cytotoxic T cell, a helper T cell and a subtype thereof.

23. A chimeric gene according to claim 1 wherein said endogenous protein is a lymphocyte receptor chain, a polypeptide of the TCR/CD3 complex, or a subunit of the Fc or IL-2 receptor.

The Board did not discuss the claims separately, and held that the specification failed to satisfy the written description requirement as to all of these claims.

The Capon Claims

Claims 1-10, all of the claims of the '221 patent, were held unpatentable on written description grounds. Claims 1-6 are directed to the chimeric DNA, claims 7, 8, and 10 to the corresponding cell comprising the DNA, and claim 9 to the chimeric protein:

> 1. A chimeric DNA encoding a membrane bound protein, said chimeric DNA comprising in reading frame:
>
> DNA encoding a signal sequence which directs said membrane bound protein to the surface membrane;
>
> DNA encoding a non-MHC restricted extracellular binding domain which is obtained from a single chain antibody that binds specifically to at least one ligand, wherein said at least one ligand is a protein on the surface of a cell or a viral protein;
>
> DNA encoding a transmembrane domain which is obtained from a protein selected from the group consisting of CD4, CD8, immunoglobulin, the CD3 zeta chain, the CD3 gamma chain, the CD3 delta chain and the CD3 epsilon chain; and
>
> DNA encoding a cytoplasmic signal-transducing domain of a protein that activates an intracellular messenger system which is obtained from CD3 zeta,
>
> wherein said extracellular domain and said cytoplasmic domain are not naturally joined together, and said cytoplasmic domain is not naturally joined to an extracellular ligand-binding domain, and when said chimeric DNA is expressed as a membrane bound protein in a host cell under conditions suitable for expression, said membrane bound protein initiates signaling in said host cell when said extracellular domain binds said at least one ligand.
>
> 2. The DNA of claim 1, wherein said single-chain antibody recognizes an antigen selected from the group consisting of viral antigens and tumor cell associated antigens.
>
> 3. The DNA of claim 2 wherein said single-chain antibody is specific for the HIV env glycoprotein.
>
> 4. The DNA of claim 1, wherein said transmembrane domain is naturally joined to said cytoplasmic domain.
>
> 5. An expression cassette comprising a transcriptional initiation region, the DNA of claim 1 under the transcriptional control of said transcriptional initiation region, and a transcriptional termination region.

6. A retroviral RNA or DNA construct comprising the expression cassette of claim 5.

7. A cell comprising the DNA of claim 1.

8. The cell of claim 7, wherein said cell is a human cell.

9. A chimeric protein comprising in the N-terminal to C-terminal direction:

a non-MHC restricted extracellular binding domain which is obtained from a single chain antibody that binds specifically to at least one ligand, wherein said at least one ligand is a protein on the surface of a cell or a viral protein;

a transmembrane domain which is obtained from a protein selected from the group consisting CD4, CD8, immunoglobulin, the CD3 zeta chain, the CD3 gamma chain, the CD3 delta chain and the CD3 epsilon chain; and

a cytoplasmic signal-transducing domain of a protein that activates an intracellular messenger system which is obtained from CD3 zeta,

wherein said extracellular domain and said cytoplasmic domain are not naturally joined together, and said cytoplasmic domain is not naturally joined to an extracellular ligand-binding domain, and when said chimeric protein is expressed as a membrane bound protein in a host cell under conditions suitable for expression, said membrane bound protein initiates signaling in said host cell when said extracellular domain binds said at least one ligand.

10. A mammalian cell comprising as a surface membrane protein, the protein of claim 9.

In addition, claims 5, 6, 7, and 8 of Capon's '046 patent were held unpatentable. These claims are directed to chimeric DNA sequences where the encoded extracellular domain is a single-chain antibody containing ligand binding activity.

The Board Decision

The Board presumed enablement by the specifications of the '221 patent and '994 application of the full scope of their claims, and based its decision solely on the ground of

failure of written description. The Board held that neither party's specification provides the requisite description of the full scope of the chimeric DNA or encoded proteins, by reference to knowledge in the art of the "structure, formula, chemical name, or physical properties" of the DNA or the proteins. In the Board's words:

> We are led by controlling precedent to understand that the full scope of novel chimeric DNA the parties claim is not described in their specifications under 35 U.S.C. §112, first paragraph, by reference to contemporary and/or prior knowledge in the art of the structure, formula, chemical name, or physical properties of many protein domains, and/or DNA sequences which encode many protein domains, which comprise single-chain proteins and/or DNA constructs made in accordance with the plans, schemes, and examples thereof the parties disclose.

Bd. op. at 4. As controlling precedent the Board cited Regents of the University of California v. Eli Lilly & Co., 119 F.3d 1559 (Fed. Cir. 1997); Fiers v. Revel Co., 984 F.2d 1164 (Fed. Cir. 1993); Amgen, Inc. v. Chugai Pharmaceutical Co., 927 F.2d 1200 (Fed. Cir. 1991); and Enzo Biochem, Inc. v. Gen-Probe, Inc., 296 F.3d 1316 (Fed. Cir. 2002). The Board summarized its holding as follows:

> Here, both Eshhar and Capon claim novel genetic material described in terms of the functional characteristics of the protein it encodes. Their specifications do not satisfy the written description requirement because persons having ordinary skill in the art would not have been able to visualize and recognize the identity of the claimed genetic material without considering additional knowledge in the art, performing additional experimentation, and testing to confirm results.

Bd. op. at 89.

DISCUSSION

Eshhar and Capon challenge both the Board's interpretation of precedent and the Board's ruling that their descriptions are inadequate. Both parties explain that their

chimeric genes are produced by selecting and combining known heavy- and light-chain immune-related DNA segments, using known DNA-linking procedures. The specifications of both parties describe procedures for identifying and obtaining the desired immune-related DNA segments and linking them into the desired chimeric genes. Both parties point to their specific examples of chimeric DNA prepared using identified known procedures, along with citation to the scientific literature as to every step of the preparative method.

The parties presented expert witnesses who placed the invention in the context of prior knowledge and explained how the descriptive text would be understood by persons of skill in the field of the invention. The witnesses explained that the principle of forming chimeric genes from selected segments of DNA was known, as well as their methods of identifying, selecting, and combining the desired segments of DNA. Dr. Eshhar presented an expert statement wherein he explained that the prior art contains extensive knowledge of the nucleotide structure of the various immune-related segments of DNA; he stated that over 785 mouse antibody DNA light chains and 1,327 mouse antibody DNA heavy chains were known and published as early as 1991. Similarly Capon's expert Dr. Desiderio discussed the prior art, also citing scientific literature:

> The linker sequences disclosed in the '221 patent (col. 24, lines 4 and 43) used to artificially join a heavy and light chain nucleic acid sequence and permit functional association of the two ligand binding regions were published by 1990, as were the methods for obtaining the mature sequences of the desired heavy and light chains for constructing a SAb (Exhibit 47, Batra et al., J., Biol. Chem., 1990; Exhibit 48, Bird et al., Science, 1988; Exhibit 50, Huston et al., PNAS, 1988; Exhibit 51, Chaudhary, PNAS, 1990, Exhibit 56, Morrison et al., Science, 1985; Exhibit 53, Sharon et al., Nature 1984).

Desiderio declaration at 4 ¶11.

Both parties stated that persons experienced in this field would readily know the structure of a chimeric gene made of a first segment of DNA encoding the single-chain variable region of an antibody, and a second segment of DNA encoding an endogenous protein. They testified that re-analysis to confirm these structures would not be needed in order to know the DNA structure of the chimeric gene, and that the Board's requirement that the specification must reproduce the "structure, formula, chemical name, or physical properties" of these DNA combinations had been overtaken by the state of the science. They stated that where the structure and properties of the DNA components were known, reanalysis was not required.

Eshhar's specification contains the nucleotide sequences of sixteen different receptor primers and four different scFv primers from which chimeric genes encoding scFvR may be obtained, while Capon's specification cites literature sources of such information. Eshhar's specification shows the production of chimeric genes encoding scFvR using primers, as listed in Eshhar's Table I. Capon stated that natural genes are isolated and joined using conventional methods, such as the polymerase chain reaction or cloning by primer repair. Capon, like Eshhar, discussed various known procedures for identifying, obtaining, and linking DNA segments, accompanied by experimental examples.

The Board did not dispute that persons in this field of science could determine the structure or formula of the linked DNA from the known structure or formula of the components.

The Board stated that "controlling precedent" required inclusion in the specification of the complete nucleotide sequence of "at least one" chimeric gene. Bd. op. at 4. The Board also objected that the claims were broader than the specific examples. Eshhar and

Capon each responds by pointing to the scientific completeness and depth of their descriptive texts, as well as to their illustrative examples. The Board did not relate any of the claims, broad or narrow, to the examples, but invalidated all of the claims without analysis of their scope and the relation of claim scope to the details of the specifications.

Eshhar and Capon both argue that they have set forth an invention whose scope is fully and fairly described, for the nucleotide sequences of the DNA in chimeric combination is readily understood to contain the nucleotide sequences of the DNA components. Eshhar points to the general and specific description in his specification of known immune-related DNA segments, including the examples of their linking. Capon points similarly to his description of selecting DNA segments that are known to express immune-related proteins, and stresses the existing knowledge of these segments and their nucleotide sequences, as well as the known procedures for selecting and combining DNA segments, as cited in the specification.

Both parties argue that the Board misconstrued precedent, and that precedent does not establish a *per se* rule requiring nucleotide-by-nucleotide re-analysis when the structure of the component DNA segments is already known, or readily determined by known procedures.

The Statutory Requirement

The required content of the patent specification is set forth in Section 112 of Title 35:

> §112 ¶1. The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full,

> clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

The "written description" requirement implements the principle that a patent must describe the technology that is sought to be patented; the requirement serves both to satisfy the inventor's obligation to disclose the technologic knowledge upon which the patent is based, and to demonstrate that the patentee was in possession of the invention that is claimed. See Enzo Biochem, 296 F.3d at 1330 (the written description requirement "is the quid pro quo of the patent system; the public must receive meaningful disclosure in exchange for being excluded from practicing the invention for a limited period of time"); Reiffin v. Microsoft Corp., 214 F.3d 1342, 1345-46 (Fed. Cir. 2000) (the purpose of the written description requirement "is to ensure that the scope of the right to exclude . . . does not overreach the scope of the inventor's contribution to the field of art as described in the patent specification"); In re Barker, 559 F.2d 588, 592 n.4 (CCPA 1977) (the goal of the written description requirement is "to clearly convey the information that an applicant has invented the subject matter which is claimed"). The written description requirement thus satisfies the policy premises of the law, whereby the inventor's technical/scientific advance is added to the body of knowledge, as consideration for the grant of patent exclusivity.

The descriptive text needed to meet these requirements varies with the nature and scope of the invention at issue, and with the scientific and technologic knowledge already in existence. The law must be applied to each invention that enters the patent process, for each patented advance is novel in relation to the state of the science. Since the law is applied to each invention in view of the state of relevant knowledge, its application will vary

with differences in the state of knowledge in the field and differences in the predictability of the science.

For the chimeric genes of the Capon and Eshhar inventions, the law must take cognizance of the scientific facts. The Board erred in refusing to consider the state of the scientific knowledge, as explained by both parties, and in declining to consider the separate scope of each of the claims. None of the cases to which the Board attributes the requirement of total DNA re-analysis, i.e., Regents v. Lilly, Fiers v. Revel, Amgen, or Enzo Biochem, require a re-description of what was already known. In Lilly, 119 F.3d at 1567, the cDNA for human insulin had never been characterized. Similarly in Fiers, 984 F.2d at 1171, much of the DNA sought to be claimed was of unknown structure, whereby this court viewed the breadth of the claims as embracing a "wish" or research "plan." In Amgen, 927 F.2d at 1206, the court explained that a novel gene was not adequately characterized by its biological function alone because such a description would represent a mere "wish to know the identity" of the novel material. In Enzo Biochem, 296 F.3d at 1326, this court reaffirmed that deposit of a physical sample may replace words when description is beyond present scientific capability. In Amgen Inc. v. Hoechst Marion Roussel, Inc., 314 F.3d 1313, 1332 (Fed. Cir. 2003) the court explained further that the written description requirement may be satisfied "if in the knowledge of the art the disclosed function is sufficiently correlated to a particular, known structure." These evolving principles were applied in Noelle v. Lederman, 355 F.3d 1343, 1349 (Fed. Cir. 2004), where the court affirmed that the human antibody there at issue was not adequately described by the structure and function of the mouse antigen; and in University of Rochester v. G.D. Searle & Co., 358 F.3d 916, 925-26 (Fed.

Cir. 2004), where the court affirmed that the description of the COX-2 enzyme did not serve to describe unknown compounds capable of selectively inhibiting the enzyme.

The "written description" requirement must be applied in the context of the particular invention and the state of the knowledge. The Board's rule that the nucleotide sequences of the chimeric genes must be fully presented, although the nucleotide sequences of the component DNA are known, is an inappropriate generalization. When the prior art includes the nucleotide information, precedent does not set a *per se* rule that the information must be determined afresh. Both parties state that a person experienced in the field of this invention would know that these known DNA segments would retain their DNA sequences when linked by known methods. Both parties explain that their invention is not in discovering which DNA segments are related to the immune response, for that is in the prior art, but in the novel combination of the DNA segments to achieve a novel result.

The "written description" requirement states that the patentee must describe the invention; it does not state that every invention must be described in the same way. As each field evolves, the balance also evolves between what is known and what is added by each inventive contribution. Both Eshhar and Capon explain that this invention does not concern the discovery of gene function or structure, as in Lilly. The chimeric genes here at issue are prepared from known DNA sequences of known function. The Board's requirement that these sequences must be analyzed and reported in the specification does not add descriptive substance. The Board erred in holding that the specifications do not meet the written description requirement because they do not reiterate the structure or formula or chemical name for the nucleotide sequences of the claimed chimeric genes.

Claim Scope

There remains the question of whether the specifications adequately support the breadth of all of the claims that are presented. The Director argues that it cannot be known whether all of the permutations and combinations covered by the claims will be effective for the intended purpose, and that the claims are too broad because they may include inoperative species. The inventors say that they have provided an adequate description and exemplification of their invention as would be understood by persons in the field of the invention. They state that biological properties typically vary, and that their specifications provide for evaluation of the effectiveness of their chimeric combinations.

It is well recognized that in the "unpredictable" fields of science, it is appropriate to recognize the variability in the science in determining the scope of the coverage to which the inventor is entitled. Such a decision usually focuses on the exemplification in the specification. See, e.g., Enzo Biochem, 296 F.3d at 1327-28 (remanding for district court to determine "[w]hether the disclosure provided by the three deposits in this case, coupled with the skill of the art, describes the genera of claims 1-3 and 5"); Lilly, 119 F.3d at 1569 (genus not described where "a representative number of cDNAs, defined by nucleotide sequence, falling within the scope of the genus" had not been provided); In re Gostelli, 872 F.2d 1008, 1012 (Fed. Cir. 1989) (two chemical compounds were insufficient description of subgenus); In re Smith, 458 F.2d 1389, 1394-95 (CCPA 1972) (disclosure of genus and one species was not sufficient description of intermediate subgenus); In re Grimme, 274 F.2d 949, 952 (CCPA 1960) (disclosure of single example and statement of scope sufficient disclosure of subgenus).

Precedent illustrates that the determination of what is needed to support generic claims to biological subject matter depends on a variety of factors, such as the existing

knowledge in the particular field, the extent and content of the prior art, the maturity of the science or technology, the predictability of the aspect at issue, and other considerations appropriate to the subject matter. See, e.g., In re Wallach, 378 F.3d 1330, 1333-34 (Fed. Cir. 2004) (an amino acid sequence supports "the entire genus of DNA sequences" that can encode the amino acid sequence because "the state of the art has developed" such that it is a routine matter to convert one to the other); University of Rochester, 358 F.3d at 925 (considering whether the patent disclosed the compounds necessary to practice the claimed method, given the state of technology); Singh v. Brake, 317 F.3d 1334, 1343 (Fed. Cir. 2002) (affirming adequacy of disclosure by distinguishing precedent in which the selection of a particular species within the claimed genus had involved "highly unpredictable results").

It is not necessary that every permutation within a generally operable invention be effective in order for an inventor to obtain a generic claim, provided that the effect is sufficiently demonstrated to characterize a generic invention. See In re Angstadt, 537 F.2d 498, 504 (CCPA 1976) ("The examples, both operative and inoperative, are the best guidance this art permits, as far as we can conclude from the record"). While the Board is correct that a generic invention requires adequate support, the sufficiency of the support must be determined in the particular case. Both Eshhar and Capon present not only general teachings of how to select and recombine the DNA, but also specific examples of the production of specified chimeric genes. For example, Eshhar points out that in Example 1 of his specification the FcRγ chain was used, which chain was amplified from a human cDNA clone, using the procedure of Kuster, H. et al., J. Biol. Chem., 265:6448-6451 (1990), which is cited in the specification and reports the complete sequence of the FcRγ

chain. Eshhar's Example 1 also explains the source of the genes that provide the heavy and light chains of the single chain antibody, citing the PhD thesis of Gideon Gross, a co-inventor, which cites a reference providing the complete sequence of the Sp6 light chain gene used to construct the single-chain antibody. Eshhar states that the structure of the Sp6 heavy chain antibody was well known to those of skill in the art and readily accessible on the internet in a database as entry EMBL:MMSP6718. Example 5 at page 54 of the Eshhar specification cites Ravetch et al., J. Exp. Med., 170:481-497 (1989) for the method of producing the CD16α DNA clone that was PCR amplified; this reference published the complete DNA sequence of the CD16α chain, as discussed in paragraph 43 of the Eshhar Declaration. Example 3 of the Eshhar specification uses the DNA of the monoclonal anti-HER2 antibody and states that the N29 hybridoma that produces this antibody was deposited with the Collection Nationale de Cultures de Microorganismes, Institut Pasteur, Paris, on August 19, 1992, under Deposit No. CNCM I-1262. It is incorrect to criticize the methods, examples, and referenced prior art of the Eshhar specification as but "a few PCR primers and probes," as does the Director's brief.

Capon's Example 3 provides a detailed description of the creation and expression of single chain antibody fused with T-cell receptor zeta chain, referring to published vectors and procedures. Capon, like Eshhar, describes gene segments and their ligation to form chimeric genes. Although Capon includes fewer specific examples in his specification than does Eshhar, both parties used standard systems of description and identification, as well as known procedures for selecting, isolating, and linking known DNA segments. Indeed, the Board's repeated observation that the full scope of all of the claims appears to be "enabled" cannot be reconciled with the Board's objection that only a "general plan" to

combine unidentified DNA is presented. See In re Wands, 858 F.2d 731, 736-37 (Fed. Cir. 1988) (experimentation to practice invention must not be "undue" for invention to be considered enabled).

The PTO points out that for biochemical processes relating to gene modification, protein expression, and immune response, success is not assured. However, generic inventions are not thereby invalid. Precedent distinguishes among generic inventions that are adequately supported, those that are merely a "wish" or "plan," the words of Fiers v. Revel, 984 F.2d at 1171, and those in between, as illustrated by Noelle v. Lederman, 355 F.3d at 1350; the facts of the specific case must be evaluated. The Board did not discuss the generic concept that both Capon and Eshhar described -- the concept of selecting and combining a gene sequence encoding the variable domain of an antibody and a sequence encoding a lymphocyte activation protein, into a single DNA sequence which, upon expression, allows for immune responses that do not occur in nature. The record does not show this concept to be in the prior art, and includes experimental verification as well as potential variability in the concept.

Whether the inventors demonstrated sufficient generality to support the scope of some or all of their claims, must be determined claim by claim. The Board did not discuss the evidence with respect to the generality of the invention and the significance of the specific examples, instead simply rejecting all the claims for lack of a complete chimeric DNA sequence. As we have discussed, that reasoning is inapt for this case. The Board's position that the patents at issue were merely an "invitation to experiment" did not distinguish among the parties' broad and narrow claims, and further concerns enablement more than written description. See Adang v. Fischhoff, 286 F.3d 1346, 1355 (Fed. Cir.

2002) (enablement involves assessment of whether one of skill in the art could make and use the invention without undue experimentation); In re Wright, 999 F.2d 1557, 1561 (Fed. Cir. 1993) (same). Although the legal criteria of enablement and written description are related and are often met by the same disclosure, they serve discrete legal requirements.

The predictability or unpredictability of the science is relevant to deciding how much experimental support is required to adequately describe the scope of an invention. Our predecessor court summarized in In re Storrs, 245 F.2d 474, 478 (CCPA 1957) that "[i]t must be borne in mind that, while it is necessary that an applicant for a patent give to the public a complete and adequate disclosure in return for the patent grant, the certainty required of the disclosure is not greater than that which is reasonable, having due regard to the subject matter involved." This aspect may warrant exploration on remand.

In summary, the Board erred in ruling that §112 imposes a *per se* rule requiring recitation in the specification of the nucleotide sequence of claimed DNA, when that sequence is already known in the field. However, the Board did not explore the support for each of the claims of both parties, in view of the specific examples and general teachings in the specifications and the known science, with application of precedent guiding review of the scope of claims.

We remand for appropriate further proceedings.

VACATED AND REMANDED